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DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR DETERMINATION OF IMPURITIES IN GRANISETRON BY USING HPLC AND UPLC

Ashlesha B. Kolhe, Dr. L. Hingane, Gulshan Rathi

Student, Assistant Professor, Assitant Professor
Department of Pharmaceutical Analysis,
Aditya Pharmacy College Beed 431122, Maharashtra, India

Abstract : **It Deals with an introduction giving a brief account of various aspects to be considered for the development of new UPLC and HPLC methods for the assay of pharmaceutical substances and their formulations in single dose (three compounds) and in combination (four compounds, only by UPLC and HPLC). The introduction includes a brief account on pharmaceutical substances and their formulations in the present investigation and general information and methodology for the development of new methods using UPLC and HPLC.**

The information given under includes a brief study on UPLC and HPLC components (Solvent delivery systems, solvent degassing system, Gradient elution devises, sample introduction systems and detectors along with column packing materials inclusive of bonded phase). System performance (System suitability)and method performance calculations such as relative retention, theoretical plates, height equivalent to theoretical plates (HETP), capacity factor, resolution, peak symmetry (Tailing factor), Validation of Analytical methods (As per ICH guidelines, for parameters- System Precision, Specificity, Method Precision, Recovery(Accuracy), Linearity, Robustness and Ruggedness) used in the present investigation. It gives a brief account of chemical name, structure, literature on physic chemical properties and mode of action, literature on available physicochemical methods reported for Granisetron. There is lack of RP-UPLC methods along with complicated process for sample preparation in case of RP-UPLC methods. Taking all these views of the drug into consideration, the author has developed a simple stability indication UPLC method for the quantitative estimation of Granisetron. A simple, accurate and reproducible reverse phase UPLC method was developed for the estimation of Granisetron in bulk drugs and formulations. The optimized method consists of mobile phase pH 6.5 ammonium acetate buffer and Acetonitrile in gradientelution mode with a run time of 4 minutes and a flow rate of 0.4 mL/min. UV detection was carried out at a wavelength of 305 nm with an injection volume of 2 µL. Acquity BEHC18 (1.7 µm, 100 mm *2.1 mm) column. The retention time of Granisetron was found to be 1.75 minutes. The developed method was validated as per ICH Q2A (R1) guideline. The proposed UPLC method was linear over the range of 10.22-30.50 µg/mL, the correlation coefficient was found to be 0.999. Relative standard deviation for method precision was found to be 0.32 % and intermediate precision was found to be 0.34 %. Limit of Detection was found to be 0.25 µg/mL and Limit of Quantification was found to be 0.77 µg/mL, respectively.

IndexTerms **- Component,formatting,style,styling,insert.**

1.0 Introduction:

1.1 Impurity profiling of pharmaceutical products

Impurity is defined as any substance coexisting with the original drug, such as starting material or intermediates or that is formed, due to any side reactions [1].

Impurities can be of three types:

(1) Impurities closely related to the product and coming from the chemical or from the biosynthetic route itself.

(2) Impurities formed due to spontaneous decomposition of the drug during the storage or on exposure to extreme conditions.

(3) The precursors that may be present in the final product as impurities. Impurities present in excess of 0.1% should be identified and quantified by selective methods. The suggested structures of the impurities can be

synthesized and will provide the final evidence for their structures, previously determined by spectroscopic methods. Therefore, it is essential to know the structure of these impurities in the drug substance in order to alter the reaction condition and to reduce the amount of impurity to an acceptable level. Isolation, identification and quantification of impurities help us in various ways, to obtain a pure substance with less toxicity and, safety in drug therapy. Quantitative determination of these impurities could be used as a method for the quality control and validation of drug substances. Regulatory authorities such as USFDA (United States Food and Drug Administration) and EMA (European Medicines Agency) insist on the impurity profiling of drugs. Impurities in new drug substances can be addressed from two perspectives:-

(1) The chemical aspect which includes classification and identification of impurities, report generation, listing of impurities in specifications, and a brief discussion of analytical procedures.
(2) The safety aspect which includes specific guidance for quantifying impurities present at substantially lower levels in batches of new drug substance used in clinical studies.

1.1.1 Quality, safety and efficacy of drug

Safety and efficacy of pharmaceuticals are two fundamental issues of importance in drug therapy. The safety of a drug is determined by its pharmacological toxicological profile as well as the adverse effects caused by the impurities in bulk and dosage forms [2]. The impurities in drugs often possess unwanted pharmacological or toxicological effects by which any benefit from their administration may be outweighed. Therefore, it is quite obvious that the products intended for human consumption must be characterized as completely as possible. The quality and safety of a drug is generally assured by monitoring and controlling the impurities effectively.

1.1.2 Impurity profile

There is no precise definition for impurity profile. It gives an account of impurities present in it. Impurity profile is a description of the identified and unidentified impurities present in a typical batch of API (Active Pharmaceutical Ingredient) produced by a specific controlled production process. It includes the identity or some qualitative analytical designation (e.g. retention time), the range of each impurity observed, and type of each identified impurity. Impurity profile of a substance under investigation gives maximum possible types of impurities present in it. It also estimates the actual amount of different kinds of impurities present in it. The impurity profile is normally dependent upon the process or origin of the API.

1.1.3 Source of impurities in drug product

Impurities in drugs are originated from various sources and phases of the synthetic process and preparation of pharmaceuticals dosage forms. A sharp difference between the process-related impurities and degradation products is always not possible. However, majority of the impurities are characteristic of the synthetic route of the manufacturing process. Since there are several possibilities of synthesizing a drug, it is possible that the same product of different sources may give rise to different impurities.

The three important sources of impurities are:

i) Synthesis- related impurities
ii) Formulation-related impurities
iii) Degradation-related impurities

i) Synthesis-related impurities

Impurities in a drug substance or a new chemical entity (NCE) originate mainly during the synthetic process from raw materials, solvents, intermediates, and byproducts. [3] The raw materials are generally manufactured to much lower purity requirements than a drug substance. Hence, it is easy to understand why they contain a number of components that can in return affect the purity of the drug substance. Similarly, solvents used in the synthesis are likely to contain a number of impurities that may range from trace levels to significant amounts that can react with various chemicals used in the synthesis to produce other impurities. Intermediates are also generally not held to the purity level of the drug substance and thus the remarks made for the raw

materials do apply. It is not reasonably possible to theorize all by-products; as a result, any such products that may be produced in the synthesis would be hard to monitor. The "pot reaction," i.e., when the intermediates are not isolated, are convenient, economical, and time saving however, they raise havoc in terms of the generation of impurities because a number of reaction can occur simultaneously can be also encountered in single reactions where intermediate is isolated. The final intermediate is generally controlled in the pharmaceutical synthesis by conducting regulatory impurity testing. This typically entails residual solvents or process impurities. It is important to remember that this step is the last major source of potential impurities; therefore, it is very desirable that the methods used for the analysis at this stage be rigorous. It should be remembered that base-to-salt or acid-to-salt conversion could also generate new impurities.

ii) Formulation related-impurities

A number of impurities in a drug product can rise out of interaction with excipients used to formulate a drug product. Furthermore, in the process of formulation, a drug substance is subjected to a variety of conditions that can be lead to its degradation or other deleterious reactions. For example, if heat is used for drying or for other reasons, it can facilitate degradation of thermally liable drug substances. Solutions and suspensions are potentially prone to degradation that is due to hydrolysis. These reactions can also occur in the dosage form in a solid state, such as in case of capsules and tablets, when water or another solvent has been used for granulation. Not only can the water used in the formulation contribute its own impurities, it can also provide a ripe situation for hydrolysis and metal catalysis. Similar reactions are possible in other solvents that may be used. Oxidation is possible for easily oxidized materials if no precautions are taken. Similarly, light-sensitive materials can undergo photochemical reactions.

iii) Degradation related-impurities

A number of impurities can be produced because of API degradation or other interactions on storage Therefore it is very important to conduct stability studies to predict, evaluate, and ensure drug product safety. Stability studies include evaluation of stability of API, pre-formulation studies to evaluate compatibility of API and excipients to determine its stability in the formulation matrix, accelerated stability evaluations of the test or final drug product, stability evaluations via kinetic studies and projection of expiration date, routine stability studies of drug products in marketed, sample or dispensed package under various conditions of temperature, light, and humidity. The stability studies under various exaggerated conditions of temperature, humidity, and light can help us to determine what potential impurities can be produced by degradation reactions. It is important to establish a viable stability program to evaluate impurities. A good stability program integrates well the scientific considerations with regularity requirements.

1.1.4 Classification of impurities

Impurities can be classified as Organic impurities (process- and drug-related), Inorganic impurities and Residual solvents. [4-6] Organic impurities may arise during the manufacturing process and/or storage of the new drug substance. These include starting materials, by-products, intermediates, degradation products, reagents, ligands, and catalysts. Inorganic impurities include reagents, ligands and catalysts, heavy metals or other residual metals, inorganic salts, filter aids, charcoal etc. Residual Solvents are organic or inorganic liquids used as vehicle in the synthesis of drug substance. Since these are, generally of known toxicity, the selection of appropriate controls can be accomplished easily.

Table 1.1: Description of impurity types and their sources

Sr.No.	Impurity Type	Impurity Source
1.	Process-related drug substance	Organic Starting material Intermediate By-product Impurity in starting material
2.	Process-related drug product	Organic or Inorganic Reagents, catalysts etc.
3.	Degradation drug substance or product	Organic Degradation products
4.	Degradation drug product	Organic Excipients interaction

1.1.5 ICH Guidelines for impurity profile

Impurities in new drug substances and drug products are dealt with new approaches to quantification and qualification. Regulatory requirements for the identification, quantification and control of impurities in drug substances and their formulated products are now being explicitly defined, particularly through the ICH [7](International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use).. This document is intended to provide guidance for registration applications on the content and qualification of impurities in new drug substances produced by chemical syntheses and not

previously registered in a member state. Biological/biotechnological, peptide, oligonucleotide, radiopharmaceutical, fermentation and semi-synthetic products derived there from, herbal products, and crude products of animal or plant origin are not covered under it.

Table 1.2 ICH guidelines for identification and qualification of impurities in bulk drugs and formulation

Dose	Threshold for	
	Identification (%)	Qualification (%)
< 1 mg	1.0	1.0
1-10 mg	0.5	1.0
10-100 mg	.2	0.5
100 mg – 2 gm.	0.1	0.5
> 2g	0.1	0.1

1.1.6 Characterization of impurities:

It is important that the authentic sample should be used for estimations, when it is available. If the estimations indicate that a given impurity content is greater than 0.1% then it must be characterized as per the FDA requirements [9-12]. Hyphenated methods such as gas chromatography, mass spectroscopy, or liquid chromatography, mass spectrometry or the number of other chromatographic-spectroscopic configuration are perfectly suitable for initial characterization of the impurities.

Characterization methods

The Characterization of impurities is generally achieved by the following:

1. Ultraviolet (UV)
2. Infra-Red Spectroscopy (IR)
3. Nuclear Magnetic Resonance (NMR)
4. Mass Spectroscopy (MS)

1. UV (Ultraviolet)

UV at a single wavelength provides selectivity of analysis. However, with the availability of diode array detectors (DAD), it is now possible to get sufficient simultaneous information at various wavelengths to ensure greater selectivity.

2. IR (Infrared spectrophotometry)

Infrared spectrophotometer provides specific information on some functional groups that allow quantification and selectivity. However, low–level detectability is frequently a problem that may require more involved approaches to circumvent the problem.

3. NMR (Nuclear Magnetic Resonance)

The ability of NMR (Nuclear Magnetic Resonance) to provide information regarding the specific bonding structure and stereochemistry of molecules which is of pharmaceutical interest has made it a powerful analytical instrument for structural elucidation. The ability of NMR- based diffusion coefficient determination to distinguish between monomeric and dimeric substances was validated using a standard mixture of authentic materials containing both monomers and dimers. Unfortunately, NMR has traditionally been used as a less sensitive method compared to other analytical techniques. Conventional sample requirements for NMR are on the order of 10mg, as compared with Mass Spectroscopy, which requires less than 1mg.

4. MS (Mass spectroscopy)

MS has an increasingly significant impact on the pharmaceutical development process over the past several decades. Advances in the design and efficiency of the interfaces that directly connect separation techniques with Mass Spectrometers have afforded new opportunities for monitoring, characterizing, and quantification of drug related substances in active pharmaceutical ingredients and pharmaceutical formulations. Single method fails to provide the necessary selectivity; orthogonal coupling of chromatographic techniques such as HPLC-TLC and HPLC-CE (High Performance Liquid chromatography coupled with Capillary Electrophoresis), or coupling of chromatographic separations with information rich spectroscopic methods such as HPLC-MS or HPLC-NMR may need to be contemplated, but hopefully only as a development tool rather than a tool for routine QC (Quality control) use.

Hyphenated Methods

i) LC-MS-MS
ii) HPLC-DAD-MS
iii) HPLC-DAD-NMR-MS
iv) GC-MS
v) LC-MS

An example of reverse-phase LC-MS analysis in gradient elution with two distinct soft ionization techniques is the Atmospheric Pressure Ionization with Electro spray Source (API-ESI) and the chemical ionization of d-allethrine.The popularity of LCMS-MS systems for complex mixture analysis of thermally labile and biologically relevant molecules, vizmosapride, is largely attributed to the "soft nature" of Atmospheric Pressure Chemical Ionization (APCI) and Atmospheric Pressure Ionization (APPI). HPLC-DAD-MS (HPLC coupled with a Diode Array UV Detector and a Mass Spectrometer) and such other techniques are almost routinely used. NMR has now been added to this combination to provide HPLC-DAD- NMRMS capabilities in instruments.

1.1.7 Isolation of impurities

For isolating impurities, if the above mentioned instrumental methods are not available, it becomes essential to synthesise these impurities and isolate them by using the following methods. For example, when hyphenated methods such as LC-MS-MS are not suitable or do not provide unambiguous characterization, it may be necessary to isolate impurities for further confirmation of structure has been established, these impurities can be synthesized by a suitable route.

The following methods have been used for isolation of impurities:

i) Liquid-liquid extraction
ii) Accelerated solvent extraction
iii) Supercritical fluid extraction
iv) Column chromatography
v) Flash chromatography
vi) Thin-layer chromatography
vii) Gas chromatography
viii) High-pressure liquid chromatography
ix) Capillary electrophoresis
x) Solid-phase extraction

Isolation should be initiated based on simple extraction or partition methods. It may be possible to extract impurities selectively on the basis of acidity, basicity or neutrality. The extraction process usually involves liquid-liquid extraction, where one phase is an organic phase i.e., nonpolar. By appropriate adjustment of the pH of the aqueous solution, one can extract acidic, basic, or neutral impurities. Further separations can be made by chromatographic methods.

1.1.8 General scheme for drug impurity profiling

Highly sophisticated instrumentation such as mass spectrophotometer attached to a Gas Chromatography or HPLC are inevitable tools in the identification of minor components (drugs, impurities, degradation products, metabolites) in various matrices.NMR spectroscopy which involves complete structure elucidation, which may require the isolation of larger components (usually by preparative HPLC). Since Mass and NMR are very expensive and in the latter case very time consuming UV rapid scanning using the diode-array detector attached to HPLC is better alternative. But the UV-HPLC method to determine impurity profile only, provided the impurity is spectro-photometrically active. Although the successful application of HPLC/DAD (Diode-Array UV Detectors) in the identification of the above mentioned minor components is restricted to those cases where the components is spectrophotometrically active and its spectrum differs sufficiently from that of the main components (parents drugs) and from other small components, this technique can be successfully used in the impurity profiling of drugs.

1.1.9 Acceptance criteria for impurities

For newly synthesized drug substances, the specification should include acceptance criteria for impurities. Stability studies, chemical development studies, and routine batch analyses can be used to predict those impurities likely to occur in the commercial product. [12-14] A rationale for the inclusion or exclusion of impurities in the specification should be included. This rationale should include a discussion of the impurity profiles observed in clinical development batches together with a consideration of the impurity profile of batches manufactured by the proposed commercial process. For impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the quantitation or detection limit of the analytical methods should be commensurate with the level at which the impurities need to be controlled.

A general acceptance criterion of not more than 0.1 % for any unspecified impurity should be included. Acceptance criteria should be set based on the data generated on actual batches of the drug substance, allowing sufficient latitude to deal with normal manufacturing and analytical variation and the stability characteristics of the drug substance. Although normal manufacturing variations are expected, significant variation in batch to batch impurity levels can indicate that the manufacturing process of the drug substance is not adequately controlled and validated. The acceptance criteria should include limits for organic impurities (each specified identified impurity, each specified unidentified impurity at or above 0.1%, any unspecified impurity with a limit of not more than 0.1%, total impurities) residual solvents and inorganic impurities. If data are not available to qualify the proposed specification level of an impurity, studies to obtain such data may be needed (when the usual qualification threshold limits given below are exceeded).

According to ICH, based on maximum daily dose the qualification threshold is as follows: For a maximum daily dose of 2g/day, qualification threshold is 0.15 % or 1 mg per day intake (whichever is lower) and for MDD of 2g/day, it is 0.05%. In the case of unsuccessful identification with standard samples, the most reasonable way to determine the structure of impurity starts with the investigation of the UV spectra, easily obtainable with the aid of the diode array detector in the case of HPLC and the quantification with the help of densitometer. In exceptional cases, with full knowledge of the synthesis of drug substance, the structure of the impurity can be generated on the basis of NMR spectral data. If the information obtained from the UV spectrum is not sufficient, the next step in the procedure of impurity profiling is to take the mass spectrum of the impurity.

The major disadvantage of this method is the volatility and thermal stability problems of the impurities. The use of derivatization reactions widely used in GC/MS analysis is problematic because the side-products of the derivatization reaction can be confused with the impurities. The next step in the impurity profiling is the synthesis of the material with the proposed structure. The possibilities of spectroscopic techniques in drug impurity profiling without chromatographic separation are also worth mentioning. Spectra obtained by using high-resolution, highly sensitive NMR spectrometers and mass spectrometers with APCI /ESI (Electromagnetic Source Imaging) facilities are suitable to provide a fingerprint picture regarding the purity of the sample.

1.1.10 Separation methods

The following other separation methods can also be used:

i) Thin-layer chromatography (TLC)
ii) Gas chromatography (GC)
iii) High-pressure liquid chromatography (HPLC)
iv) Capillary electrophoresis (CE)
v) Supercritical fluid chromatography (SFC)

A broad range of compounds can be resolved using TLC by utilizing a variety of different plates and mobile phases [15]. The primary difficulties related to this method are limited resolution, detection and ease of quantification. The greatest advantages are the ease of use and low cost. Gas chromatography is a very useful technique for quantification. It can provide the desired resolution, selectivity, and ease of quantification. However, the primary limitation is that the sample must be volatile or has to be made volatile by derivatization. This technique is very useful for organic volatile impurities. High-pressure liquid chromatography is frequently referred to as high-performance liquid chromatography today. Both of these terms can be abbreviated as HPLC, and they are used interchangeably by chromatographers. This is a useful technique with applications that have been significantly extended for the pharmaceutical chemist by the use of a variety of detectors such as fluorescence, electrometric, MS, etc. Capillary electrophoresis is a useful technique when very low quantities of samples are available and high resolution is required. The primary difficulty is assuring reproducibility of the injected samples.

Supercritical fluid chromatography offers some of the advantages of GC in terms of separations, in that volatility of the sample is not of paramount importance. This technique is still evolving, and its greatest application has been found in the extraction of samples.

1.1.11 Development of HPLC methods for monitoring of process impurities in drug substances

Currently, the vast majority of drug-related impurity determinations are performed by HPLC. It offered the desired sensitivity for trace level determinations with a high degree of automation. A wide variety of stationary phases and operation modes make HPLC applicable to all drug classes. The typical detection limits for drug related impurities by HPLC are 0.1% or lower and this can be routinely met in the majority of circumstances using conventional UV detectors. During the period of review, HPLC was used extensively in the evaluation and control of purity of chemical components used in the manufacture of bulk drugs.

A variety of approaches to establish the impurity profiles of synthetic drugs have been outlined. These methods involved the prediction of likely impurities within the synthetic process, their isolation and identification by suitable analytical techniques. [16-18] However, these are valid only for materials synthesized by routes. Any changes in the synthetic route may lead to a different profile of impurities and must be investigated accordingly. Methods based on some form of HPLC separation still outnumber all other assays as they have for over two decades. The bulk of these are relatively simple isocratic reversed-phase procedures that employ UV detection. However, in some instances, more novel approaches are often based on separation mode or the use of more selective detective systems typically diode array and fluorescence detectors or mass spectrometers. Of these, the application of coupled LC-MS continues to increase in popularity, as electrospray ionization and atmospheric pressure ionization interfaces became more reliable at higher throughput volumes. Several approaches using HPLC are appropriate for determination of impurities in bulk drug substances and their formulations.

1.2 Introduction to chromatography

Techniques related to Chromatography have been used for centuries to separate materials such as dyes extracted from plants. Russian botanist Tswett is credited with the discovery of chromatography. In 1903 he succeeded in separating leaf pigments using a solid polar stationary phase. It was not until 1930s that this technique was followed by Kuhn and Lederer as well as Reichstein and Van Euw for the separation of natural products [19, 20]. Martin and Synge were awarded the

Noble Prize for their work in 1941 in which they described liquid-liquid partition chromatography [21, 22]. Martin and Synge applied the concept of theoretical plates as a measure of chromatographic efficiency. The term “Chromatography “is derived from the two Greek words Chroma; which mean color, and the word Graphein; is to write [23, 24].

1.2.1 Chromatography in the pharmaceutical world

In the modern pharmaceutical industry, chromatography is the major and integral analytical tool applied in all stages of drug discovery, development, and production. The development of new chemical entities (NCEs) is comprised of two major activities: drug discovery and drug development. The goal of the drug discovery program is to investigate a plethora of compounds employing fast screening approaches, leading to generation of lead compounds and then narrowing the selection through targeted synthesis and selective screening (lead optimization). The main functions of drug development are to completely characterize candidate compounds by performing drug metabolism, preclinical and clinical screening, and clinical trials. Throughout this drug discovery and drug development paradigm, rugged analytical HPLC separation methods are developed, at each phase of development the analyses of a myriad of samples are performed to adequately control and monitor the quality of the prospective drug candidates, excipients, and final products. Effective and fast method development is of paramount importance throughout this drug development life cycle. This requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization [25].

IUPAC definition of chromatography

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in adefinite direction [26].

USP definition of chromatography

Chromatography is defined as a procedure by which solutes are separated by dynamic differential migration process in a system consisting of two or more phases, one of which moves continuously in a given direction and in which the individual substances exhibit different mobility’s by reason of difference in adsorption, partition, solubility, vapour pressure, molecular size or ionic charge density. The individual substances thus separated can be identified or determined by analytical procedures [27].

1.2.2 History of chromatography

During the 1930’s and 1940’s Chromatography progressed rapidly with several parallel developments of the earlier work which have resulted in the various chromatographic techniques we use today. A brief note on the historical developments of the main techniques is presented below [28].

1.2.2.1 Paper chromatography

Paper Chromatography was one of the earliest recognized forms of Chromatography; it was the development due to Martin and Co-Workers. Their work on partition column chromatography required an adsorbent that would retain water more efficiently than silica Gel. This led to the use of cellulose successfully over 20 Amino Acids by a two-dimensional technique using Ninhydrin to locate the spots. Although still used as a screening technique it has been overtaken by thin layer Chromatography with its higher separating efficiencies [29].

1.2.2.2 Thin Layer chromatography

The earliest Thin Layer Chromatography separations were reported by the Dutch biologist Beyerinck in 1889 and Wijsman in 1898, which used gelatin layers to separate strong acids and enzymes in malt extract respectively. TLC as we know it today, originated from the work of Izmailov and Sharaiber in 1938, who analyzed pharmaceutical tinctures, including extracts of Cinnamon, Belladonna, Foxglove by spotting samples on to a Thin Layer of Alumina adsorbent on a glass plate and applying spots of solvent to give circular chromatograms[29].

1.2.2.3 Ion exchange chromatography

The use of Ion Exchange Chromatography was first reported by Taylor and Urey in 1938 to separate Lithinium and Potassium isotopes using Zeolite resins. The application of high performance liquid chromatography techniques to IEC in the 1980's led to the development of high performance ion chromatography, LC or HPLC. High efficiency ion exchange columns and sensitive conductivity detectors enable samples containing ppm levels of anions or cations to be separated in min. Ion exchange is a process wherein a solution of an electrolyte is brought into contact with an ion exchange resin and active ions on the resin are replaced by ions (ionic species) of similar charge from the analyte solution [29].

1.2.2.4 Gel permeation chromatography/size exclusion/gel filtration

GPC uses material with a controlled pore size stationary phase. The discovery of Flodin and Porath in 1958 of a suitable cross-linked gel formed by the reaction of dextran with epicholorohydrin provided the break-through. Analysis by GPC of polymeric materials has revolutionized molecular weight analysis and preparative separation of high-molecular weight synthetic polymers. [29]

1.2.2.5 Affinity chromatography

Affinity Chromatography is a relatively recent development to Porath et al. in 1967, specifically for the analysis of biological samples. The stationary phase is a peptide or protein which has a specific binding affinity for a particular analyte. It is covalently bonded to ligands such as a nucleic acid or an enzyme on an inert open matrix of cellulose or agar. Only analyte with specific affinity for the ligand will be retarded and separated. Affinity Chromatography is used for a range of biochemical analyses including the separation of protein molecules [29].

1.2.2.6 Gas chromatography

Gas chromatography is one of the most extensively used tools for quantitative analysis of drugs in biologic samples. Gas chromatography offers the advantages of speed, sensitivity, resolution and simplicity for both qualitative and quantitative drug analysis [29]. There are broadly three modes in which GC is carried out:

- Gas liquid chromatography using a packed column with the liquid stationary phase coated onto inert support particles.

- Capillary column GC where open tubular columns are used with the liquid or solid stationary phase coated onto the inner walls of the column tubing referred to as wall coated, porous layer and surface coated open tubular columns.

- Gas solid chromatography using a packed column with the solid surface of the particles forming the stationary phase. E.g. Alumina or a Cross linked polymer.

1.2.2.7 Supercritical fluid chromatography

Supercritical fluid chromatography has features that are common to both GC and HPLC. A supercritical fluid such as carbon dioxide with its low viscosity and low diffusion coefficients is used as the mobile phase with bonded phase HPLC or capillary GC columns modified to withstand the high pressures and temperatures. In GC flame ionization detector is used [30].

1.2.2.8 Capillary (zone) electrophoresis

Capillary electrophoresis is named for a relatively new group of related techniques which have been attracting increasing interest in which separation of analyte species is achieved on the basis of differential migration in an electric field through narrow bore fused silica capillary columns (25-100µm)[31]. The techniques which differ significantly in operative and separation characteristics include:

- Capillary zone electrophoresis

- Capillary gel electrophoresis

- Isoelectric focusing

- Isotachophoresis and

- Micellarelectrokinetic capillary chromatography

1.2.2.9 High performance liquid chromatography

High Performance Liquid Chromatography (HPLC) is one of the mostly used analytical techniques in pharmaceutical industry. Chromatographic process can be defined as separation technique involving mass-transfer between stationary and mobile phase. HPLC utilizes a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures [32].

Theory

HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing beads, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process: Hydrophobic (non-specific) interactions are the main ones in reversed-phase (RP) separations. Dipole-dipole (polar) interactions are dominant in normal phase (NP) mode. Ionic interactions are responsible for the retention in ion-exchange chromatography. All these interactions are competitive. Analyte molecules compete with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface. The weaker the eluent interaction, the longer the analyte will be retained on the surface [32].

Types of HPLC

a. Adsorption chromatography: The stationary phase is an adsorbent (like silica gel or any other silica based packing) and the separation is based on repeated adsorption-desorption steps [32].

b. Ion-exchange chromatography: The stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time [32].

c. Size exclusion chromatography: The column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate into the pores of the packing particles and elute later. This technique is also called gel filtration or gel permeation chromatography. Concerning the first type, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography. In normal phase chromatography, the stationary bed is strongly polar in nature (e.g. silica gel), and the mobile phase is nonpolar (such as n-hexane). Polar samples are thus retained on the polar surface of the column packing for longer than less polar materials. Reversed-phase chromatography is the inverse of this. The stationary bed is nonpolar in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained. Reverse phase chromatography is used for almost 90% of all chromatographic applications. Eluent polarity plays the major role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type, constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run [32].

HPLC as compared with the classical LC technique is characterized by:

a. high resolution
b. small diameter (4.6 mm), stainless steel, glass or titanium columns
c. column packing with very small (3, 5 and 10 μm) particles
d. relatively high inlet pressures and controlled flow of the mobile phase
e. continuous flow detectors capable of handling small flow rates and detecting very small amounts
f. rapid analysis

Initially, pressure was selected as the principal criterion of modern liquid chromatography and thus the name was "high pressure liquid chromatography" or HPLC. This was, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is, however, not true. In fact, high performance is the result of many factors: very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume

sample injectors, sensitive low volume detectors, and good pumping systems. Naturally, pressure is needed to permit a given flow rate of the mobile phase.

Stationary phases

HPLC separations are based on the surface interactions, and depend on the types of the adsorption sites. Modern HPLC adsorbents are the small rigid porous particles with high surface area [32].

Main adsorbent parameters are:

a. Particle size: 3 to 10 μm.

b. Particle size distribution: as narrow as possible, usually within 10% of the mean.

The last parameter in the list represents an adsorbent surface chemistry. Depending on the type of the ligand attached to the surface, the adsorbent could be normal phase (-OH, -NH2), or reversed-phase (C5, C8, C 18 CN, NH2), and anion (CH2NR3 +OH-), or cation (R-SO3 -H+) exchangers.

Mobile phases

In HPLC, the type and composition of the eluent is one of the variables influencing the separation. Despite the large variety of solvents used in HPLC, there are several common properties which include: [32]

a. Purity

b. Detector compatibility

c. Solubility of the sample

d. Low viscosity

e. Chemical inertness

For normal phase mode; solvents are mainly nonpolar, for reversed-phase; eluents are usually a mixture of water with some polar organic solvent such as acetonitrile or methanol. Size-exclusion HPLC has special requirements. SEC eluents have to dissolve polymers, but the most important is that SEC eluent has to suppress possible interactions of the sample molecule with the surface of the packing material [32].

Instrumentation of HPLC

HPLC instrumentation includes a pump, injector, column, detector and data system. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometer size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analyte and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on the recorder. Detection of the eluting components is important, and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyze the chromatographic data, computer, integrator, and other data processing equipment are frequently used [32].

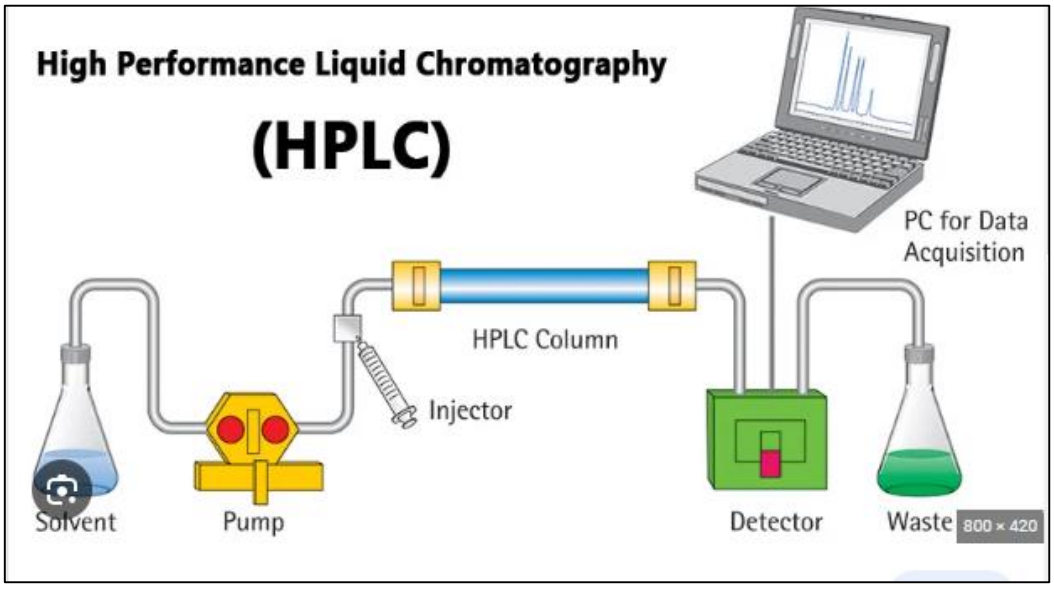

Fig. 1.1: Schematic representation of HPLC

1.3 HPLC method development guidelines

Introduction

The need to save method development time and improve accuracy is forcing today's analytical chemists to look for better, faster ways to develop stability indicating methods. Starting with HPLC columns that offer excellent reproducibility, column lifetime and sensitivity, this step-by-step protocol can save the method development chemist time and money required to establish new method. This approach is consistent with developing process.

1.3.1 Procedure (protocol) for method development

1.3.1.1Literature survey

Conduct literature survey and collect information available from the following references

- Chemical abstracts
- Analytical abstracts
- Journals
- National library of medicines etc.,

And collect the following literature from survey.

Solubility profile: Solubility of drug in different solvents at different pH conditions which is useful while selecting the diluents for standard solution and extraction solvents for test solution.

Analytical profile: Physico-chemical and spectroscopic properties, impurity and degradation profile of drug substance. Spectral profile is useful in the selection of detector wavelength for analysis, whereas degradation profile helps to develop the method for separation of all possible impurities and degradants from API.

Stability profile: Stability of the drug with storage conditions. This helps to adopt suitable and adequate precautions while handling drug substances and its solutions.

1.3.2 Selection and optimization of mobile phase

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all individual impurities and degradants from analyte (API) peak. The selection of mobile phase is done always in combination with selection of column (stationary phase). The following are the parameters, which shall be taken into consideration during the selection and optimization of mobile phase.

- Buffer, if any and its strength
- pH of buffer or pH of mobile phase
- Mobile phase composition

(a) Buffer, if any and its strength

Buffer and its strength play an important role in deciding the peak symmetries and separation. Various types of buffers can be employed for achieving the required separation. The following are some of the most commonly used ones.

- Phosphate buffers : KH_2PO_4, K_2HPO_4, Na_2HPO_4, H_3PO_4
- Acetate buffers : Ammonium acetate, Sodium acetate
- Amine buffers : Triethyl amine/ Diethyl amine
- Buffers with various ion pair reagents like Tetra Butyl ammonium hydrogen sulphate butane sulphonic acid, Hexane sulphonic acid, Heptane sulphonic acid etc.,

It is important to use the buffers with suitable strength to cope up for the injection load on the column. Otherwise, peak tailing may arise due to changes in ionic form during chromatography. The retention times also depend on the molar strength of the buffer since the molar strength is inversely proportional to retention time. Ideally, the strength of the buffer shall be adopted in between 0.05M to 0.2M. The selection of the buffer and its strength is done always in combination with selection of organic phase composition in mobile phase. The strength of the buffer can be altered if necessary to achieve the required separation. But it has to be ensured that the higher strength of the buffer shall not result in precipitation/turbidity either in mobile phase or in standard and test solution while allowed standing in bench top or in refrigerator. Experiments shall be conducted using different buffers having different strength to obtain the required separation.

The buffer having a particular strength, which gives separation of all individual impurities from API peak, shall be selected. Then strength of the buffer can be varied by about 10 to 20 % from the selected buffer strength and the effect of variation shall be studied. After reviewing the results of variation, the buffer and its strength shall be selected, this is rugged for at least 2% variation in strength.

(b) pH of the buffer or pH of the mobile phase

pH plays an important role in achieving the chromatographic separation as it controls the elution properties by controlling the ionization characteristics. Depending on the pKa, drug molecule ionises, retention time of the drug molecule changes.

Acids show an increase in retention time as the pH decreases, while bases show decrease in retention time. Experiments shall be conducted using buffers having different pH to obtain the required separation. It is important that the pH of the mobile phase should be in the range of 2.0 to 8.0, as most of the columns do not withstand a pH outside this range. This is due to fact that the siloxane linkages are cleaved below pH 2.0, while at pH values above 8.0, silica may dissolve. If a pH outside this range is found necessary, packing materials which can withstand these ranges shall be chosen. pH of the buffer, which gives separation of all individual impurities from each other and from API, shall be selected. Then pH is varied by ± 0.2 from the selected pH and effect of variation shall be studied. After reviewing the results of variation, a pH is selected which is rugged at least for ± 0.2 of the selected pH.

(c) Mobile phase composition

In reverse phase chromatography, the separation is mainly controlled by the hydrophobic interactions between drug molecule and the alkyl chains on the columns packing material. Most chromatographic separations can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are methanol and acetonitrile. Tetra hydro furan is also used but to a lesser extent. Experiments shall be conducted with mobile phase having buffers with different pH and organic phases to check for the best separation between the impurities. A drug solution having all possible known impurities can be used for checking the extent of separation with different mobile phase ratios. Alternatively solution of stressed drug substance can be used to check for the separation of impurities. A mobile phase composition which gives separation of all possible impurities and degradants from API Peak and which is rugged at least for ± 0.5 in both aqueous and organic phase shall be selected.

1.3.3 Selection of detector wavelength

Selection of detector wavelength is a critical step in finalization of the analytical method. To determine the exact wavelength, standard API is injected into chromatographic system with Photo Diode Array detector and at the wave length, which gives higher response for the compound will be selected [33].

1.3.4 Selection of column

Column plays the most important role in achieving the chromatographic separation.

The following parameters should be considered while selecting a column.

- Length and diameter of the column
- Packing material
- Size and shape of particles
- Pore size, surface area and end capping
- Percentage of carbon loading

Columns with silica as a packing material is used widely in Normal phase chromatography, where the eluent (mobile phase) is non-polar consisting of various organic solvents and the stationary phase is polar. The silanol groups on the surface of the silica give it a polar character.

In Reverse phase chromatography a wide variety of columns is available covering a wide range of polarity by cross linking the silanol groups with alkyl chains like C6, C8, C18 and Nitrile groups (-CN), Phenyl groups (-C6H6) and amino groups (-NH2).

ORDER OF THE SILICA BASED COLUMNS

I--------Non Polar--------Moderately Polar--------Polar--------I

C18 < C8 < C6 < Phenyl < Amino < Cyano< Silica

Experiments are conducted using different columns with different mobile phase to achieve best separation. A column which separates all the impurities and degradants from API peak and which is rugged with mobile phase variation is selected.

1.3.5 Selection of solvent delivery system

Chromatographic separations with single eluent i.e., all the constituents of mobile phase mixed and pumped as single eluent is called Isocratic Elution and is always preferable. However Gradient Elution is a powerful

tool in achieving separation between closely eluting compounds having different polarities. The importance of Gradient Elution is that the polarity and Ionic strength of the mobile phase can be changed during the run [34]. Gradient elution is of two types:

1. Low pressure Gradient and
2. High pressure Gradient Elution

Low pressure Gradient is one in which mobile phases are mixed at pre-determined ratios and in High pressure Gradient mobile phases are pumped at different flow rates to achieve the required composition and mixed in mixing chamber and then introduced to the column. Low pressure Gradient is opted when NMT 80 % of organic phase has to be pumped. High pressure Gradient is opted when more than 80 % of organic phase has to be pumped. While optimizing the gradient elution it is important to monitor the following:

- The graph is to be monitored so as to ensure that the overall system pressure will not cross 300 bars at any point during the run.

- Flow rate is to be physically cross checked by collecting the output from the detector during the run at different time intervals. This avoids pumping problems which may arise due to higher organic phase compositions.

1.3.6 Selection of flow rate

Flow rate is selected based on the following factors

- Retention time
- Column composition
- Separation of impurities
- Peak symmetry

Preferably flow rate shall not be more than 2.5 mL/Min. a flow rate that gives least retention times, good peak symmetries, least back pressure and better separation of impurities from API peak [35].

1.3.7 Selection of column temperature

Ambient temperature is always preferred as column temperature. However if the peak symmetry could not be achieved then the column temperature can be varied between 30 °C to 80°C. If a column temperature above 80 °C is found necessary, packing material which can withstand to that temperature shall be chosen. The increase in column temperature generally will result in reduction in peak asymmetry and peak retentions [36].

1.3.8 Selection of diluent and extraction procedure

Diluent for test preparation is selected initially based on solubility of the drug substance. It is selected in such a way that the drug substance is soluble in which the extraction is complete, due to which there won't be any interference and in which peak symmetry and resolution between impurities and API peak is found satisfactory. General methods followed for extraction are Sonication, Rotatory shaking or both. In some cases where API is not extracted by the above methods, then heating is adopted if substance is stable and it should not precipitate upon cooling to room temperature [37].

Experiments are conducted to optimize the extraction of API in the presence of excipients at different test concentrations using the diluents chosen based on solubility at different time intervals of Sonication time or rotary shaking or both. The test concentrations at which the extraction is most efficient shall be selected [37].

1.3.9 Selection of test concentration and injection volume

The test concentration is generally chosen based upon the response of API peak at the selected detector wavelength. However test concentration is finalized after it is proved that API is completely extractable at the selected test concentration. Generally an injection volume of 10 to 20 µL is recommended for estimation of API. However if the extractions are found to be difficult then the test concentration can be kept low and the injection volume can be increased up to 50 µL. But it is to be ensured that at the selected volume the column is not overloaded.

1.3.10 Establishment of stability of the solution

The assessment of stability of the solution is carried out by keeping the test solution at room temperature and at 2 °C to 8 °C. This test solution is injected along with the freshly prepared standard and % RSD of the standard solution and test solution is calculated. An acceptance criterion here is that the % RSD should not be NMT 2 % [38].

1.3.11 Establishment of system suitability

System suitability parameter has to be selected based on the Tailing factor, Plate count, Resolution, and RSD. In general resolution factor for the closely eluting compounds is selected as a system suitability requirement. If the separation of impurities from each other and from API peak is found to be satisfactory, there is no need to keep a resolution factor as system suitability parameter. In such cases only standard reproducibility and symmetry of standard peak can be adopted as a system suitability requirement [38].

1.4 UPLC Introduction:

Ultra Performance Liquid Chromatography commonly referred as UPLC, is an improvisation in three areas of analysis: chromatographic resolution, speed and sensitivity of analysis. Fine particles were used in its stationary phase thus saving time and solvent consumption. UPLC was developed basing on HPLC. HPLC contains the packed materials that are involved in separation and resolution of components in a mixture. The principle of HPLC states that as column packing particle size & length decreases its efficiency increases due to which resolution also increases. As particle size decreases to less than 2.5µm, there is a significant increase in efficiency and even it will not diminish at increased linear velocities or rates of flow in relation to the common Van Deemter equation. By using very tiny particles, speed and peak capacity (number of peaks resolved per unit time) can be increased to new limits resulting in Ultra Performance known as, UPLC. The classic separation method is HPLC (High Performance Liquid Chromatography) with features like robustness, ease of use, speed, accuracy, good selectivity and adjustable sensitivity. But in comparison with gas chromatography or the capillary electrophoresis, classic HPLC lacked its efficiency due to the low diffusion coefficients in mobile phase involving deliberate diffusion of analytes in the stationary phase and mobile phase. The Van Deemter equation states that efficiency raises with the use of smaller particles, decreased column length. But this result in swift increase of back pressure while most of the HPLC system can operate under the pressure of 200 to 400 bars only. It is this reason, short columns are filled with particles of about 2 µm to speed up the analysis without loss of effectiveness, while maintaining a good enough loss of load on columns used in HPLC.

To improve the efficiency of the HPLC separations, the subsequent actions can be taken:

- Can be used under higher temperatures
- Monolithic columns can be preferred

1.4.1 Principle:

The UPLC was based on the principle of using the stationary phase with particles of size less than 2 µm which is governed by the van Deemter equation. (HPLC columns are typically filled with particles ranging from 3 to 5 µm). van Deemter equation, is an empirical formula that describes the association between linear velocity (flow rate) and plate height (HETP or column efficiency).The Van Deemter curve, ruled by an equation with three components shows that the usable flow range for a superior efficiency with smaller particles is much greater when compared with large diameter particles.

$$H = A + B/V + CV$$

Where A, B and C are constants and v is the linear velocity, the carrier gas flow rate can be determined as follows. The term A is independent of the velocity and its represents "eddy" mixing. It is slight when the packed column particles are very tiny and even. The B term represents axial diffusion or the natural diffusion tendency of the molecules. This effect is reduced at high flow rates and so this expression is divided by v. The C term is due to kinetic resistance to equilibrium of the separation process. The kinetic resistance is the time interval involved in moving from the mobile phase to the packing stationary phase and back again. The better the flow of mobile phase is the more a molecule on the pack tends to lag behind molecules in the mobile phase and this term is proportional to v.

The arrival of UPLC is the novel instrumental system for liquid chromatography, which has the added advantage of the separation performance (by reducing dead volumes), under consistent high pressures (about 8000 to 15,000 Psi compared with 2500 to 5000 Psi in HPLC). Efficiency is directly proportional to the column length and inversely proportional to the particle size. Therefore, the column is shortened by the same factor as the particle size without loss of the resolution. The advent of UPLC resulted in the detection of additional drug metabolites and greater separation

1.4.2 Instrumentation:

Table 1.3: Instrumental Comparison between UPLC and HPLC

Characteristics	HPLC	UPLC
Particle size (µ)	3 to 5	Less than 2
Maximum backpressure(MPa)	35-40	103.5

1.4.3 The instrumentation of ACQUITY UPLC:

- Sample injection
- Solvent reservoirs
- UPLC Column
- Column manager (or) heater (or) cooler
- Detectors
- Software's
- Accessories
- Connection insight service

1.4.3.1 Sample Injection:

In UPLC sample introduction is difficult through conventional injection valves, as they may be automated or manual and are not made to work at extreme pressure. To guard the UPLC columns from extreme pressure variations, the injection route must be relatively pulse-free and the swept volume of the device also requires being minimal to lessen potential band spreading. A fast injection cycle time is required to fully capitalize on the speed handled by UPLC, which in turn require a high sample capacity and low volume injections with negligible carryover. This can increase the sensitivity of analysis. There are also direct injection approaches for biological and analytical samples.

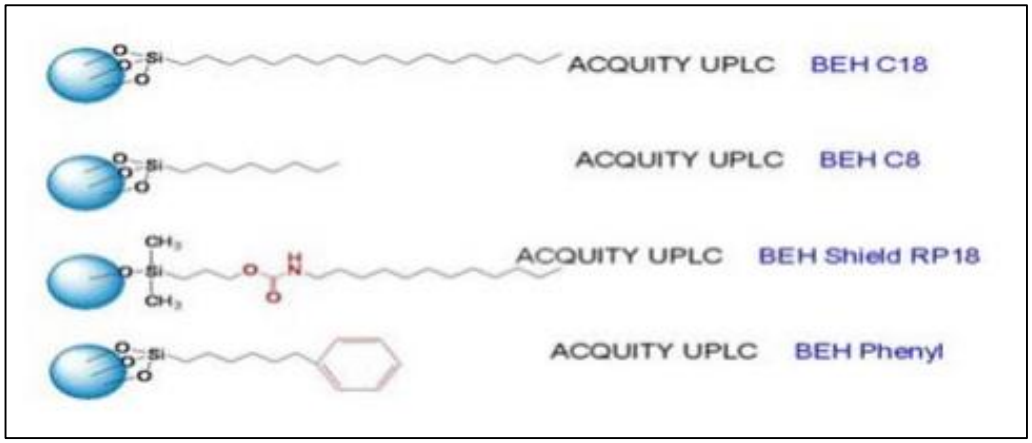

Fig. 1.2: Systemic Diagram of UPLC columns

Resolution & efficiency is improved by packing 1.7µm sized particles in the column. Generally partition of the components of a sample requires a bonded phase that offers both retention and selectivity. Four bonded phases are existing for UPLC separations: BEH C18 and C8 (straight chain alkyl columns), UPLC BEH Shield RP18 (entrenched polar group column) and UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl).UPLC column chemistry provides a different combination of hydrophobicity, silanol activity, hydrolytic stability and chemical interaction with analytes to improves separation.bvUPLC BEH C18 and C8 columns are the universal columns of choice for most of the UPLC separations which provide the widest range of pH. These columns include tri functional ligand bonding chemistries that produce superior low pH stability. This low pH stability is combined with the high pH stability of the 1.7µm sized particles to deliver the widest pH operating range. UPLC BEH Shield RP18 columns are designed to provide selectivity that complements the UPLC BEH C18 and C8 phases. UPLC BEH Phenyl columns utilize a tri functional C6 alkyl chain between the phenyl ring and the silyl functionality. This ligand, shared with the same proprietary end capping process as the UPLC BEH C18 and C8 columns, provides long column lifetimes and tremendous peak shape. This sole amalgamation of ligand and end capping on the 1.7 µm BEH particle creates a new aspect in selectivity allowing a quick match to the prevailing HPLC columns. An internal diameter (ID) of 2.1mm column is used. For utmost resolution, select 100 mm length columns and for faster analysis, and higher sample quantity, choose 50 mm

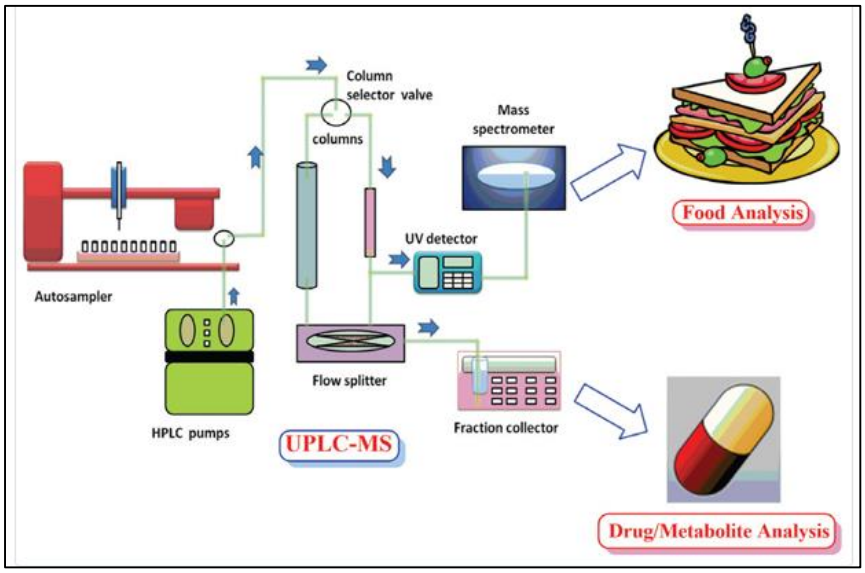

Fig. 1.3: Systemic Diagram of UPLC columns

Half-height peak widths of less than a second are obtained with 1.7µm particles, which is a challenge for the detector capacity. In order to detect an analytic peak precisely and reproducibly, the detector sampling rate must be elevated enough to capture adequate data points from the peak. The detector cell must have nominal volume to sanctuary separation efficiency. Conceptually, the sensitivity of UPLC detection must be 2-3 times greater than HPLC separations, depending on the technique used.

The ACQUITY UPLC System consists of a binary or quaternary solvent manager & sample manager together with the column heater, detector, and optional sample organizer. The binary solvent manager consists of two individual serial flow pumps to bring a parallel binary gradient programmed. There are built-in solvent specific valves to choose up to four solvents at a time. There is a 15,000-psi pressure limit (about 1000 bar) to obtain complete benefit of the sub-2µm particles.

1.4.3.3 Detectors:

The small particle chemistries utilized in the UPLC system chromatography produce very narrow peaks in analysis. Detectors used in UPLC system ACQUITY TUV, PDA, ELS and FLR and mass spectrometers collect data at faster rates to describe the resulted peaks without affecting the sensitivity or accuracy of peak measurement. These specially matched detectors operate with lower flow cell volume, minimized tubing volume and specialized fittings to control band spreading and maintain the characteristic narrowness of the peaks.

Recommended Solvents:

- Acetonitrile
- Acetonitrile and water mixtures
- Isopropanol
- Methanol
- Methanol and water mixtures
- HPLC grade water

Advantages of UPLC:

A decrease run time and increases the sensitivity, It delivers high selectivity, sensitivity and provides a dynamic range of LC analysis, Maintains high resolution, Has wider scope for Multi residual Methods, UPLC quickly quantifies the related and unrelated compounds, Use of very fine particle size results in higher analysis, It is more economical than HPLC, Solvent consumption is less, Reduces process cycle times, resulting in increased production with minimal resources.

Disadvantages of UPLC:

Increased pressure in the columns of UPLC requires more maintenance and reduces the separation. Generally in stationary phase particle size is around 2µm which is non-generable and thus it is a limitation.

Sr.No.	HPLC	UPLC
1.	HPLC typically produces broader peaks that can be characterized very well, including peak heights and peak width. HPLC also gives good data for quantification.	UPLC produces extremely sharp and detailed peaks similar to gas chromatography.
2.	Traditional HPLC lacks the resolution to separate the multiple peaks of similar components, which makes it difficult to identify or quantify those peaks.	UPLC produces more resolution and sharper peaks exhibiting higher baseline resolution thus improving granular analysis.
3.	The typical HPLC run time ranges from 20 min to as long as 40 or 50 min. HPLC run is designed for batch use or method validation which may take 30 min involving 40 to 50 injections.	In UPLC the run time is faster. That means higher pressure used with UPLC cuts which may take certainly less than ten min.
4.	The maximal pressure limit of HPLC is up to 5000 psi.	The maximum pressure that can be applied in UPLC is up to 15000 psi.
5.	HPLC analysis results can be obtained at decreased resolution	In lesser time, by using lower eluent volumes UPLC results can be obtained with increased resolution.
6.	The HPLC columns are typically filled with particle size is 3 to 5µm	The UPLC consists of stationary phase of particle size less than 2µm.

Table 1.4: Comparison between HPLC and UPLC

1.5 UPLC and HPLC method validation guidelines

Method validation is the process of proving that an analytical method is accepted for its intended purpose. For pharmaceutical methods, guidelines from the Unites States of Pharmacopeia (USP), International Conference on Harmonization (ICH) and the Food and Drug Administration (FDA) provides a frame work for performing such validations [39].

Definitions

FDA defines validation as "Establish the documented evidence which provides as high degree of assurance that a specific process will consistently produce a product of pre-determined specifications and quantity attributes" [40].

EU GMP defines validation as "action of providing in accordance with the principle of Good Manufacturing Practice (GMP), that any material, activity or system actually lead to expected result" [41].

AUSTRALIAN GMP defines validation as "the action of proving that any Material, Process, Activity, Procedure, System, Equipment's or Mechanism used in manufacture or control can and will be reliable to achieve the desire and intended result" [42].

Objective of method validation

The objective of validation is to form a basis for written procedure for production and process control, which are designed to assure that the drug products have the Identity, Quality and Purity.

Types of analytical procedures to be validated

- Identification tests.
- Quantitative tests for impurities content.
- Limit tests for the control of impurities.
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.
- Dissolution testing for drug products

- Particle size determination for drug substance.

Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (for e.g., spectrum, chromatographic behaviour, chemical reactivity) to that of a reference standard [42].

Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. These tests are intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than for a limit test.

Assay procedures are intended to measure the analyte present in a given sample. The assay represents a quantitative measurement of the major components in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected components. The same validation characteristics may also apply to assays associated with other analytical procedures (e.g., dissolution).

1.6 Validation parameters (As per ICH guidelines)

Typical validation characteristics which should be considered are [43]

Accuracy, Precision (Repeatability, Intermediate precision), Specificity, Linearity,

Detection limit, Quantitation limit, Range, Robustness, Ruggedness

Accuracy/Recovery

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Determination

In case of assay of a drug in a formulated product, accuracy may be determined by application of the analytical method to synthetic mixtures of the drug product components to which the known amount of analyte have been added within the range of the method. If it is not possible to obtain all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare results with those of a second well characterized method, the accuracy of which has been stated or defined.

ICH Requirement

ICH documents recommended that accuracy should be assessed using a minimum of 9 determinations over a minimum of three concentration levels covering the specified range i.e., three concentrations and three replicated of each concentration. For assay method, spiked samples are prepared in triplicate at three intervals over a range of 50 to 150% of the targeted concentration [44].

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation, coefficient of variation of a series of measurements [43].

Determination

The precision of an analytical method is determined by assaying a sufficient number of aliquots of homogenous lots to be able to calculate statistically valid estimates of standard deviation and Relative Standard Deviation.

ICH Requirement

ICH Documents recommended that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure i.e., three concentrations and three replicates of each concentration or using a minimum of six determinations at 100% of the test concentration [44].

Specificity

The specificity of the method corresponds to the non-interaction of the placebo with that of the active moiety [44].

Determination

Perform the test as per the test method on weight of placebo in triplicate equivalent to the amount present in weight of tablet powder.

Linearity:

The linearity of an analytical method is its ability (within a given range) to obtain the test results which are directly proportional to the concentration (amount) of analyte in the sample [44].

Determination

Linearity of an analytical procedure is established using a minimum of five concentrations. It is established initially by visual examination of a plot of signals as a function of analyte concentration or content. If there appears to be a linear relationship, test results are established by appropriate statistical methods i.e., by calculation of a regression line by the method of least squares

ICH Requirement

ICH recommends that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered. Assay of drug substance or a finished product from 25% to 150% of the test concentration [44].

Limit of detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value [44].

Determination

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as $DL = 3.3 \sigma / S$

Where

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimation of σ may be carried out in a variety of ways, for example:

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analysing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve A

Specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

(c) ICH Requirement

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification. In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit [43].

Limit of quantitation

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products [44].

Determination

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as: $QL = 10\ \sigma / S$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways including

Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

ICH Requirement

The quantitation limit and the method used for determining the quantitation limit should be presented. The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.

Range

The range of an analytical procedure is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Determination

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

ICH Requirement

- For the assay of an active substance or a finished product: normally from 80 to 120% of the test concentration;

- If assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [44].

Determination

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are:

• Stability of analytical solutions, Extraction time

In the case of liquid chromatography, examples of typical variations are

• Influence of variations of pH in a mobile phase, Influence of variations in mobile phase composition, Different columns (different lots and/or suppliers), Temperature, Flow rate.

Ruggedness

Ruggedness is not addressed in the ICH documents. Its definition has been replaced by reproducibility, which has the same meaning as ruggedness. As defined by the USP it is the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst.

Determination

Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

System suitability testing

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated [44].

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2.1 DRUG PROFILE:

2.1.1 GRANISETRON

Granisetron is a serotonin 5-HT3 receptor antagonist used as an antiemetic to treat nausea and vomiting following chemotherapy and Radiotherapy. Its main effect is to reduce the activity of the vagus nerve, which is a nerve that activates the vomiting center in the medulla oblongata. It does not have much effect on vomiting due to motion sickness. This drug does not have any effect on dopamine receptors or muscarinic receptors. Granisetron has had little effect on blood pressure, heart rate or ECG [1-5].

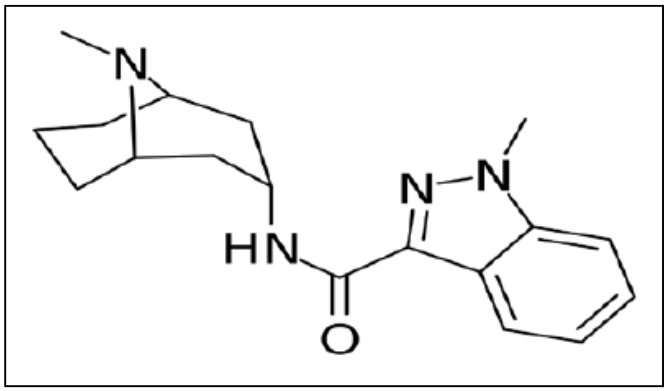

Fig. 2.1: Chemical structure of Granisetron

IUPAC Name : 1-Methyl-N-((1R, 3r, 5S)-9-Methyl-9-aza bicycle [3.3.1] nonan-3-yl-1H- indazole-3-carboxamide
Empirical Formula: C18H24N4O
Molecular Weight: 312.42 g/mol
Mechanism of action:
Granisetron is a potent, selective antagonist of 5-HT3 receptors. The antiemetic activity of the drug is brought about through the inhibition of 5-HT3 receptors present both centrally (medullary chemoreceptor zone) and peripherally (GI tract). This inhibition of 5-HT3 receptors in turn inhibits the visceral afferent stimulation of the vomiting center, likely indirectly at the level of the area postrema, as well as through direct inhibition of serotonin activity within the area postrema and the chemoreceptor trigger zone.
PHARMACOKINETICS:
Granisetron is a selective inhibitor of type 3 serotonergic (5-HT3) receptors. Granisetron has little or no affinity for other serotonin receptors, including 5-HT 1, 5-HT 1A, 5-HT 1B/C, or 5-HT 2; for alpha 1 -, alpha 2 -, or beta adrenoreceptors; for dopamine D 2 receptors; for histamine H 1 receptors; for benzodiazepine receptors; for picrotoxin receptors; or for opioid receptors. In most human studies, granisetron has had little effect on blood pressure, heart rate, or electrocardiogram (ECG). The drug is structurally and pharmacologically related to ondansetron, another selective inhibitor of 5-HT3 receptors. The serontonin 5-HT3 receptors are located on the nerve terminals of the vagus in the periphery, and centrally in the chemoreceptor trigger zone of the area postrema. The temporal relationship between the emetogenic action of emetogenic drugs and the release of serotonin, as well as the efficacy of antiemetic agents suggest that chemotherapeutic agents release serotonin from the enterochromaffin cells of the small intestine by causing degenerative changes in the GI tract. The serotonin then stimulates the vagal and splanchnic nerve receptors that project to the medullary vomiting center, as well as the 5-HT3 receptors in the area postrema, thus initiating the vomiting reflex, causing nausea and vomiting.
Absorption: Absorption of is rapid and complete, though oral bioavailability is reduced to about 60% as a result of first pass metabolism.
Metabolism and Elimination:
Primarily hepatic; undergoes N -demethylation and aromatic ring oxidation followed by conjugation. Animal studies suggest that some of the metabolites may have 5-HT 3 receptor antagonist activity.
Indication:
For the prevention of nausea and vomiting associated with initial and repeat courses of emetogenic cancer therapy (including high dose cisplatin), post operation, and radiation (including total body irradiation and daily fractionated abdominal radiation). Literature survey reveals that HPLC-UV [5-6], LC-MS/MS [7], UPLC/MS-MS [8-10] methods have been reported for the estimation of Granisetron in bulk, finished formulations and in biological samples. Granisetron Hydrochloride is official in United States pharmacopeia (USP), British Pharmacopeia (BP) and Indian Pharmacopeia (IP) for its qualitative and quantitative determination. Recently stability indicating HPLC method for the determination of Granisetron in bulk and its degraded products was reported. The objective of the present work is to develop a stability indicating UPLC method and validated as per ICH and USP validation guidelines [11-15] for the estimation of Granisetron in quality control laboratories with respect to specificity, limit of detection and quantification, linearity, precision, ruggedness and robustness with shorter run time. Granisetron is highly soluble BCS class-III drug and is formulated as a film coated tablets with 1 mg and 2 mg strengths and Resolutions. Granisetron is marketed under the trade name KYTRIL [16-20].

3.0 INSTRUMENT AND REAGENTS:

Table 3.1: List of Reagents

Sr. No.	Name	Make	Grade
1.	Ammonium acetate	Merck, India ltd, Mumbai	AR
2.	Acetonitrile	Merck, India ltd, Mumbai	HPLC
3.	Acetic acid	Rankem, India ltd, Mumbai	HPLC
4.	Water	Milli-Q water purification system, (Milford, USA)	Millipore
5.	Hydrochloric acid	Merck, India ltd, Mumbai	AR
6.	Sodium hydroxide	Merck, India ltd, Mumbai	AR
7.	Potassium dihydrogen phosphate	Merck, India ltd, Mumbai	AR
8.	Hydrogen peroxide	Merck, India ltd, Mumbai	AR
9.	0.45 µm Nylon filter	Rankem	NA

Table 3.2 : List of Instruments

Sr.No.	Instrument Name	Make and Model	Manufacturer/supplier
1.	UPLC	Waters e2695	Waters, USA
2.	Semi Micro Balance	Sartorius ME235P	Sartorius Mechatronics, India
3.	Analytical Balance	Shimadzu AUX220	Shimadzu corporation, Kyoto, Japan
4.	Sonicator	Ultrasonic Cleaner Power sonic 420	Hwashin technology co, Korea
5.	Centrifuge	Eppendorf Centrifuge 5810	Chennai, India
6.	Vacuum oven	Wadegati;WIL-190	Mumbai, India

4.0 PREPARATION OF SOLUTIONS:

4.1 PREPARATION OF SYSTEM SUITABILITY SOLUTION:

The standard solution was exposed to sun light for 8 H or exposure the solution to UV light for 24 h. During the light exposure Granisetron converts into its related compound-C. The resolution between the peak of Granisetron and its related compound-C should be not less than 3.5.

4.2 PREPARATION OF STANDARD SOLUTION:

A working standard stock solution of Granisetron Hydrochloride was prepared by dissolving standard equivalent to 20 mg of Granisetron into 100 ml volumetric flask, to this added 30 ml of diluent and sonicated for 10 min at a temperature not exceeding 20°C. Allowed the solution to attain room temperature and then diluted to the volume with diluent to have a solution with concentration of 200 µg/ml.

4.3 PREPARATION OF SAMPLE SOLUTION:

Weighed 20 tablets and determined the average weight of the tablets and crush them to fine powder by using mortar and pestle. Transfer crushed powder equivalent to 20 mg of Granisetron into 100 ml volumetric flask and added 30ml of diluent and sonicated in ultrasonic bath for 20 min with intermediate shaking at a temperature not more than 20°C. Allowed the flask to attain room temperature and diluted to the volume with

diluent. Filter the solution through 0.45 µm nylon membrane filter by discarding 4 ml of filtrate and injected the same solution (0.2 mg/ml).

4.4 PREPARATION OF PLACEBO SOLUTION:

Weighed accurately 10 mg of placebo powder into 100 ml volumetric flask added 30 ml of diluent and sonicated in ultrasonic bath for 20 min with intermediate shaking at a temperature not more than 20°C. Allowed the flask to attain room temperature and diluted to the volume with diluent. Filter the solution through0.45 µm nylon membrane filter by discarding 4 ml of filtrate and injected the same solution.

5.0 ANALYTICAL METHOD DEVELOPMENT

5.1 SELECTION OF WAVELENGTH:

Standard concentration of about 200 µg/mL of Granisetron was prepared and scanned the solution from 200.0 nm to 400.0 nm .

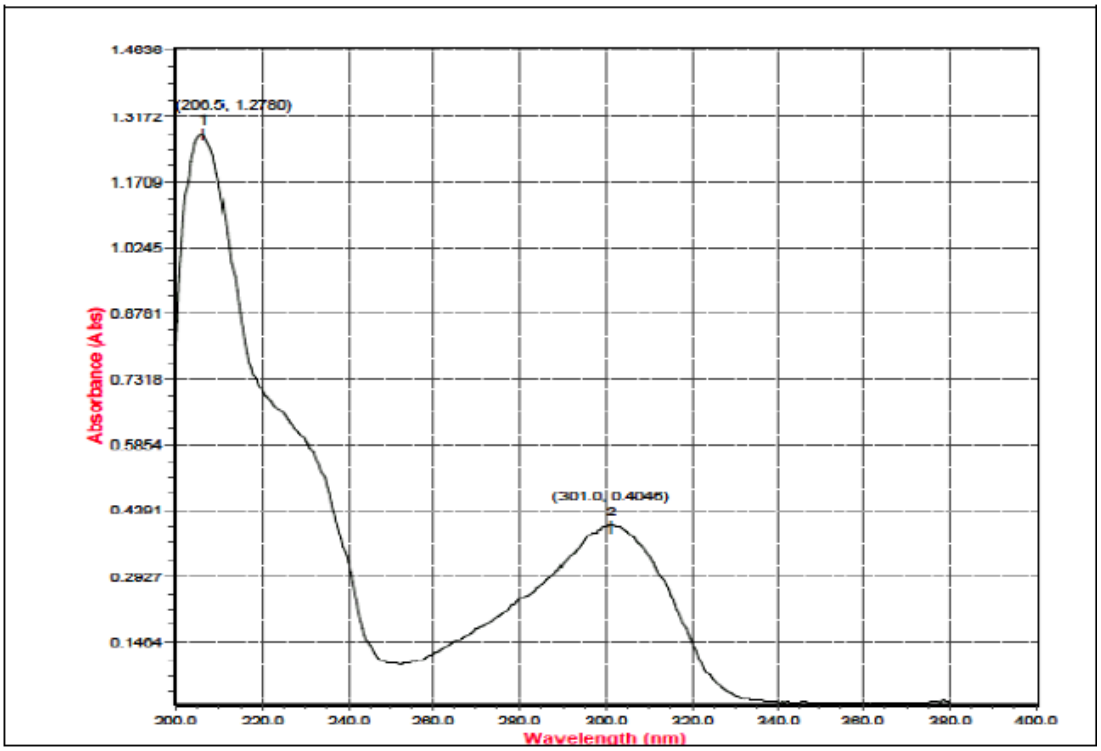

Fig. 5.1 : A Typical Spectrum of Granisetron

5.2 TRIALS:

Selection of Mobile phase:

Liquid chromatograph - Mass compatible, high UV transparent and desired baseline with well separation of impurities.

Selection of Stationary phase:

Since the compound is mid polar in nature the capacity of retention is little more in C18 as compared to C8. The 1.7 µm Ethylene Bridged Hybrid [BEH] particle is one of the key enablers behind UPLC Technology. Due to the intrinsic chemical stability of hybrid particle technology, a wider usable pH range [pH 1-12] can be employed, enabling a versatile and robust separation technology for method development.

Providing unprecedented levels of peak asymmetry, efficiency, and chemical stability, the 1.7 µm ACQUITY UPLC BEH C18 and C8 Columns are versatile, high-performance separation columns suitable for a diverse range of analytes. With the ability to operate between pH 1-12, these trifunctionally-bonded alkyl columns can employ the power of pH to impact the retention, selectivity, and sensitivity of ionizable compounds while delivering exceptional low- and high-pH stability.

Method development Trial-1:

The standard solution were prepared and injected in to system with the following experimental conditions.

Stationary phase : BEH C8 (150 mm x 2.1 mm, 1.7 µ)
Mobile phase-A : 0.05 % TFA in water (% v/v)
Mobile phase-B : 0.05 % TFA in Acetonitrile (% v/v)
Diluent : Water : Acetonitrile (50:50) (% v/v)
Gradient (Time-min/%B) : 0/50,2/70,2.5/80,3.1/50,4/50

Detection : 305
Injection Volume : 1.0 µL
Temperature : 35 ºC
Run time : 4 min
Flow Rate : 0.6 mL/min

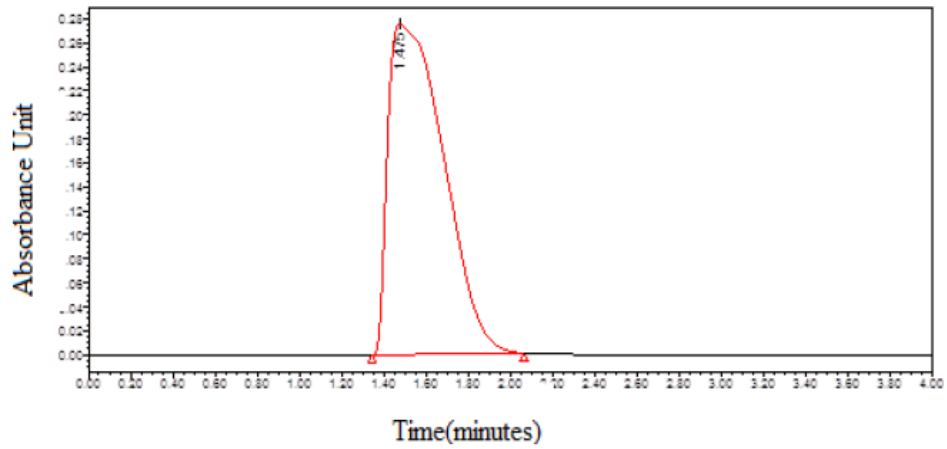

Fig. 5.2 : Chromatogram of Trial-1

Observation: Distorted peak was observed.

Method development Trial-2:

Stationary phase : BEH C8 (150 mm x 2.1 mm, 1.7 µ)
Mobile phase-A : 0.05 % OPA in water (% v/v)
Mobile phase-B : 0.05 % OPA in Acetonitrile (% v/v)
Diluent : Water: Acetonitrile (50:50) (% v/v)
Gradient (Time-min/%B) : 0/50,2/60,2.5/80,3.1/50,4/50
Detection : 305
Injection Volume : 2.0 µL
Temperature : 25 ºC
Run time : 4 min
Flow Rate : 0.6 mL/min

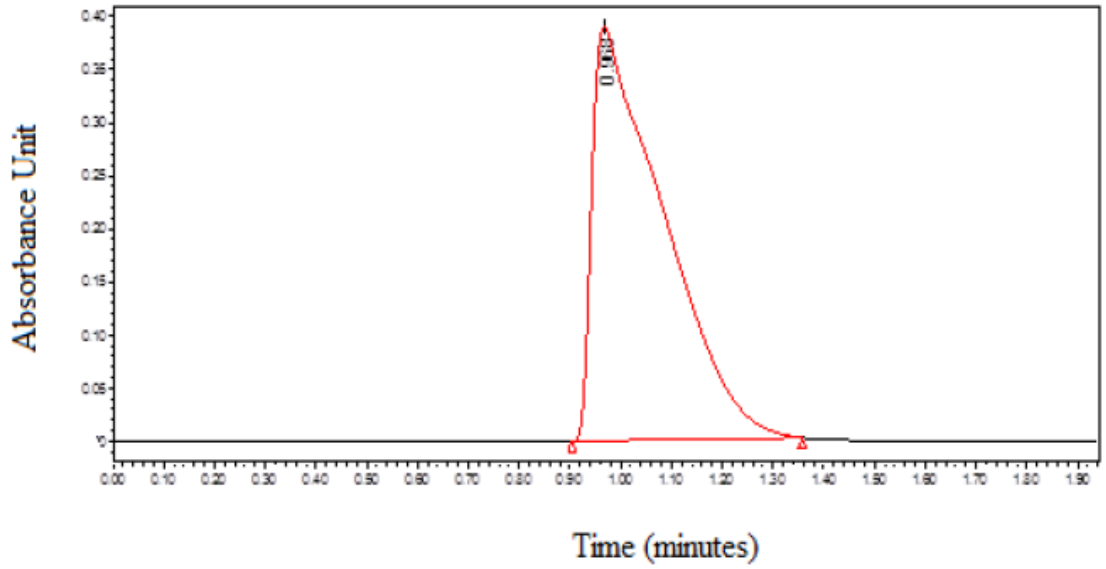

Fig. 5.3 : Chromatogram of Trial-2

Observation: In this trial also observed distorted peak.

Method development Trial-3:

Stationary phase : BEH C18 (150 mm x 2.1 mm, 1.7 µ)
Mobile phase-A : 0.05 % OPA in water (% v/v)
Mobile phase-B : 0.05 % OPA in Acetonitrile (% v/v)
Diluent : Water: Acetonitrile (50:50) (% v/v)

Gradient (Time-min/%B) : 0/50,2/60,2.5/80,3.1/50,4/50
Detection : 305
Injection Volume : 1.0 µL
Temperature : 25 ºC
Run time : 4 min
Flow Rate : 0.6 mL/min

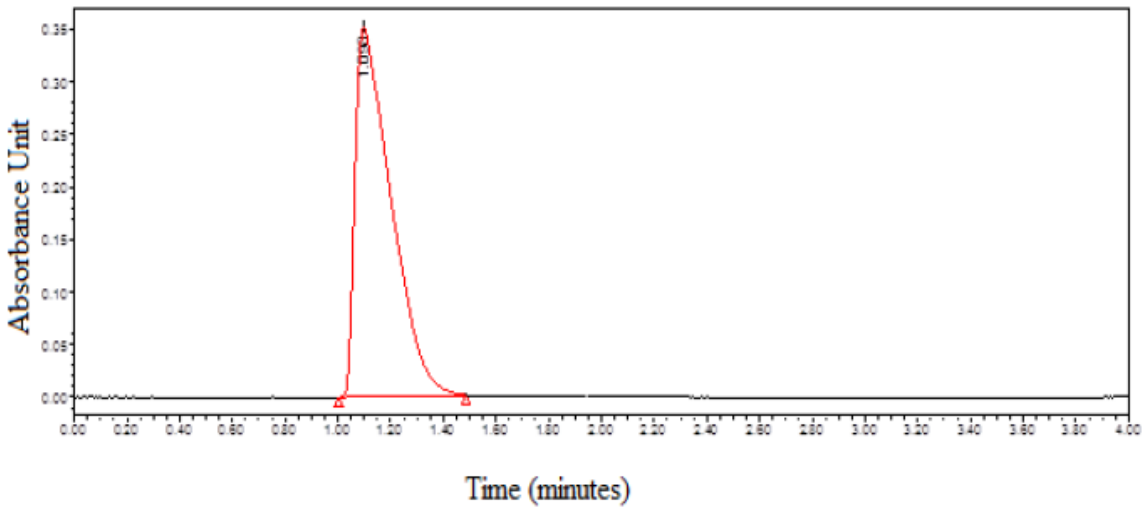

Fig. 5.4: Chromatogram of Trial-3

Observation: Good peak shape but Tailing observed.

Optimized method conditions:

Stationary phase : BEH C18 (150 mm x 2.1 mm, 1.7 µ)
Mobile phase-A : 10 mM Ammonium acetate in Water
Mobile phase-B : Acetonitrile (100%)
Diluent : Water : Acetonitrile (50:50) (% v/v)
Gradient (Time-min/%B) : 0/50,2/70,3/70,3.1/50,4/50
Detection : 305
Injection Volume : 2.0 µL
Temperature : 40 ºC
Run time : 4 min
Flow Rate : 0.4 mL/min
Preparation of buffer : Dissolved accurately 0.77 g of ammonium acetate in 1000 ml of milli-q water and mixed well further adjusted the pH of the solution to 6.5 ± 0.05with acetic acid. The buffer solution was filtered through 0.45 µm nylon membrane filter.

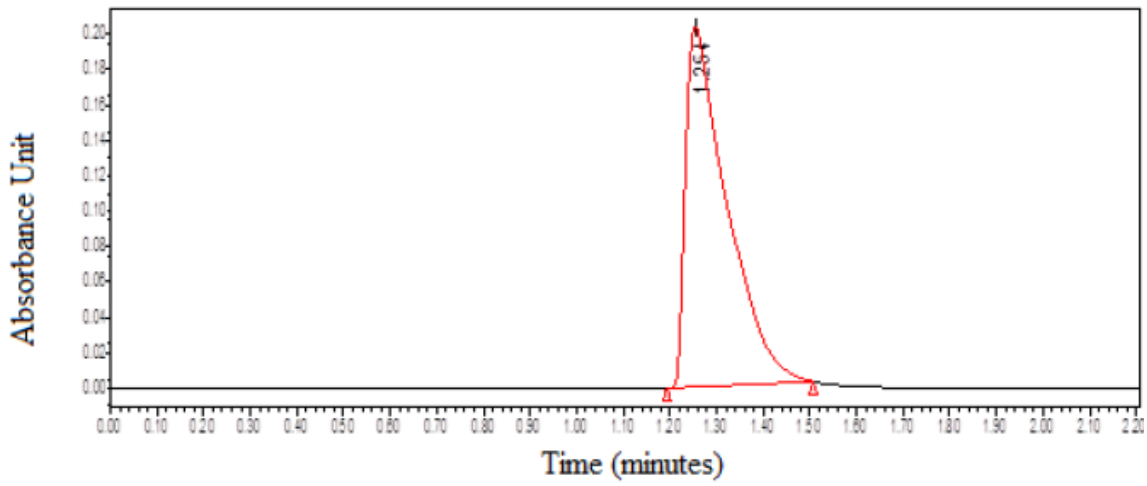

Fig. 5.5: Chromatogram of Trial-4

Observation: Good peak shape was observed.

6.0 RESULTS AND DISCUSSION

The present work was aimed at the analytical method development and validation for the estimation of Granisetron in tablet dosage form by RP-UPLC method. As there was no stability indicating UPLC method available for the determination of Granisetron in bulk and drug product with proper peak shapes and shorter run time. The objective of the current method is to separate all the potential impurity peaks arise during the forced degradation study from Granisetron peak. For the optimization of the UPLC method, forced degradation sample was taken as reference. Initial trials were taken on pH-4.5Ammonium format buffer with acetonitrile as mobile phase and test concentration of 200 ppm in mobile phase was injected in which there was no clear separation between Granisetron and its related compound-C. Further trials were taken by varying the pH value of the mobile phase buffer from 4.5 to 6.5. i.e., ammonium acetate buffer was selected as a mobile phase as it LC-MS compatible and also having maximum buffering capacity at its pKa. Forced degradation samples were injected and found that all the four known impurities (Impurity-A, Impurity-B, Impurity-C and Impurity-D) were separated with longer run time and broader peak shapes in isocratic mode. In order to shorten the run time gradient separation mode was optimized with satisfactory separation. Optimal separation was achieved on Acquity BEH C18 100*2.1 mm, 1.7 µm UPLC column maintained at 40°C. Gradient elution was performed using the mixture of 10 mM ammonium acetate buffer pH-6.5 (pH was adjusted with Ammonia) and acetonitrile as organic modifier at a flow rate of 0.4 mL/min. Detection was carried out at a wavelength of 305 nm. Sample cooler compartment was maintained at a temperature of 5°C. pH 6.5 10 mM Ammonium acetate buffer used as Mobile phase A and Acetonitrile used as Mobile phase B.

6.1 OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Based on the above studies, the following optimized chromatographic condition was selected for the analysis.

Gradient Program:

Time (min)	Flow rate (ml/min)	Mobile phase-A (%)	Mobile phase-B (%)
0	0.4	50	50
2	0.4	30	70
3	0.4.	30	70
3.1	0.4	50	50
4	0.4	50	50

Table 6.1 : Optimized Chromatographic Conditions

Chromatographic mode	RP-UPLC
Detector	PDA-detector
Stationary Phase	Acquity BEH C18 100*2.1 mm, 1.7 µm
Mobile phase	pH 6.5 10mM Ammonium acetate buffer used as Mobile phase A and Acetonitrile used as Mobile phase B.
Elution mode	Gradient
Detection wavelength (nm)	305
Flow rate (mL/min)	0.4
Injection volume (µL)	2
Column temperature (ºC)	40
Sample temperature (ºC)	5

With the selection of optimal condition, the standard and sample solutions were injected and the chromatograms recorded.

7.0 ANALYTICAL METHOD VALIDATION:

Analytical method validation is the process of demonstrating that analytical procedures are suitable for their intended use. More specifically analytical method validation is matter of establishing documented evidence that provides a high degree of assurance that a facility or operation will consistently produce product meeting a predetermined specification.

The following parameters were considered:

System Suitability, Specificity, Precision, Accuracy (Recovery),Linearity, Range,
Robustness, Solution stability, LOD & LOQ

7.1 SYSTEM SUITABILITY TESTING:

System suitability solution was prepared and injected to evaluate the system suitability of the method and found that Granisetron was separated from its eight known impurities with good resolution. The developed UPLC method was found to be specific for determination of Granisetron from its known impurities namely Fig.2.10 shows separation of all the eight known impurities from Granisetron in the proposed method.

Table 7.1 : System Suitability Parameters for Optimized Chromatographic Condition

Name	RT	Area	USP Tailing	USP Plate Count
Granisetron	1.75	5285948	1.20	15193

7.2 SPECIFICITY:

7.2.1 Interference from Blank and Placebo:

Specificity is the ability of the method to measure the analyte response in presence of its potential known impurities. Specificity of the developed UPLC method for Granisetron was carried out in the presence of blank, placebo and its known impurities i.e., Imp-A, Imp-B, Imp-C Imp-D and Imp-E for the accurate measurement of Granisetron present in the sample. As a part of specificity, stress studies were carried out for Granisetron drug substance, drug product and placebo under stress conditions like oxidation, acid, base, photolytic and thermal (105°C). These stress samples were analysed using the proposed method at a concentration of 200 µg/ml of Granisetron to separate all the Granisetron impurities from Granisetron peak. In these stress conditions the peak purity test was verified for the Granisetron peak and by using diode array detector.

The specificity of the method was evaluated by verifying the peak purity of the sample. The method was found to be specific as there was no interference from blank and placebo at the retention time of main peak. No degradant peaks were observed at the retention time of Granisetron during the degradation and stability study indicates that the method is stability indicating and also purity angle was found to be less than purity threshold indicates that there was no spectral coelution for Granisetron peak in this method and also the resolution between the neighboring peak was greater than 2.0.

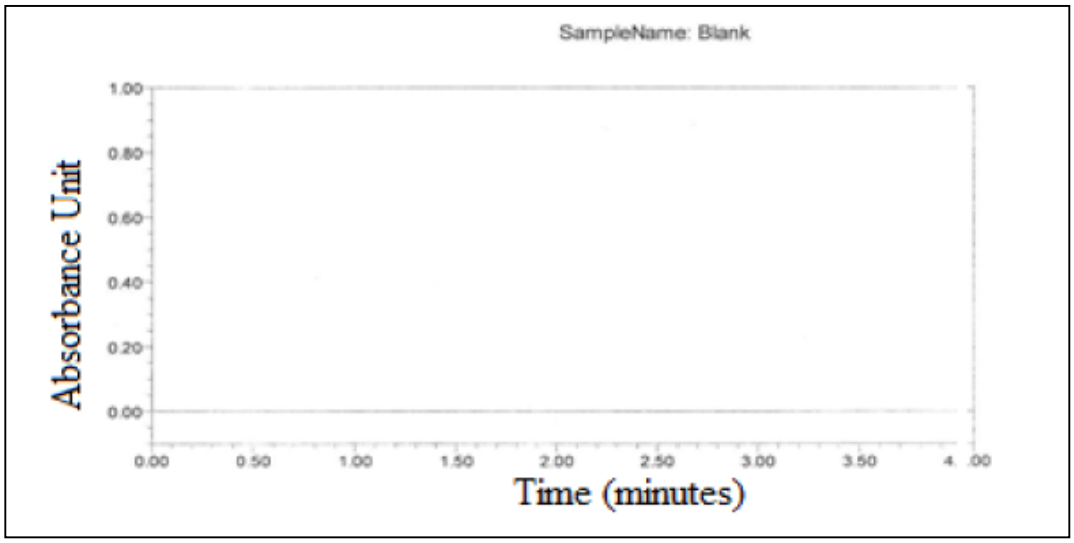

Fig. 7.2: Chromatogram of Blank

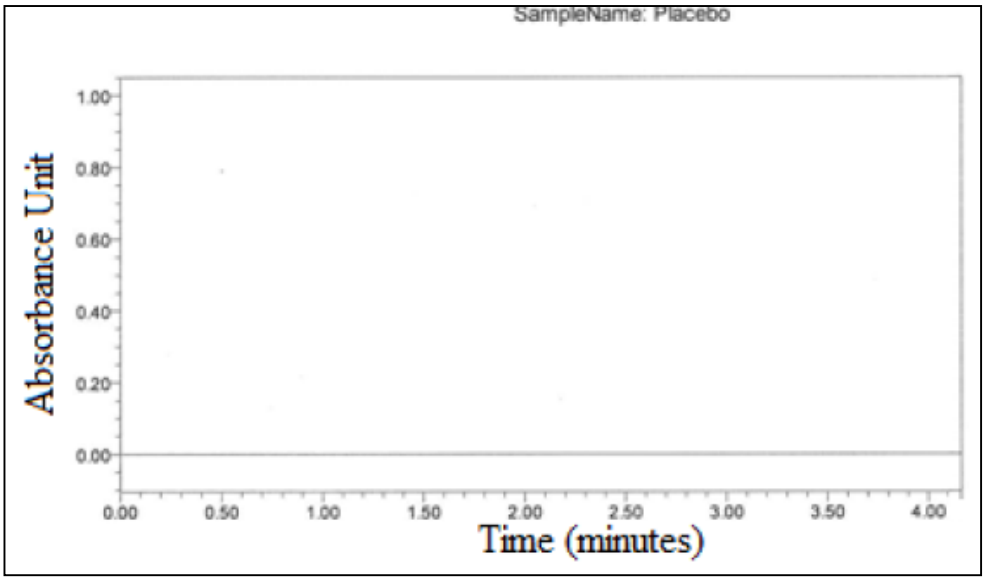

Fig. 7.3: Chromatogram of Placebo

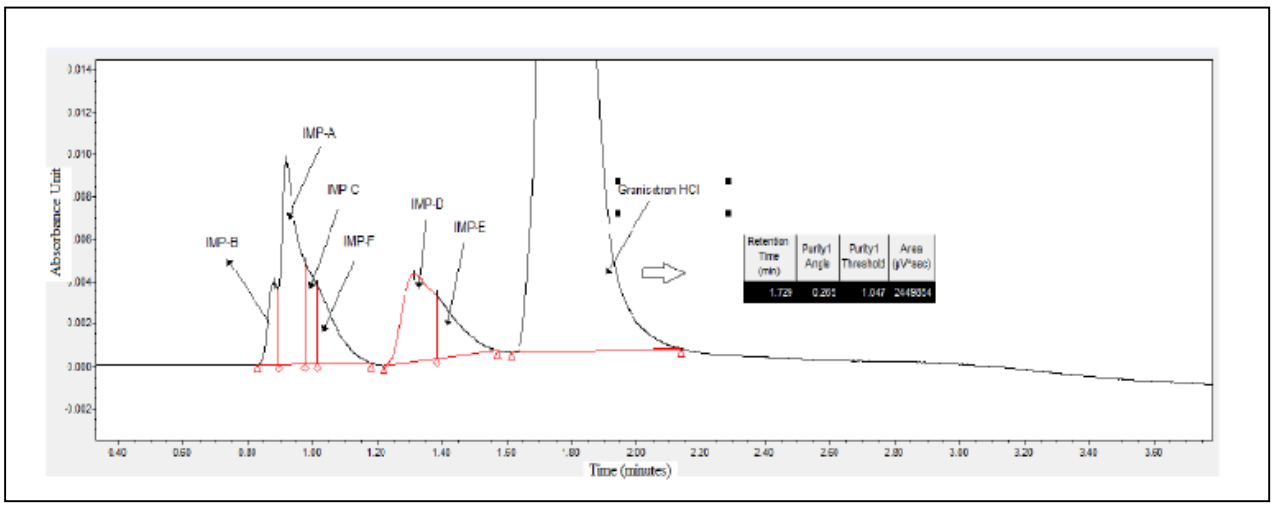

Fig. 7.4: Chromatogram of Granisetron spiked sample

7.2.2 Forced degradation studies:

Forced degradation studies were performed to establish the stability indicating power of the method. In this study Granisetron raw material, finished product and placebo were subjected to acidic, basic, peroxide, thermal and photolytic stress studies on sample concentration of 0.2 mg/ml in diluent. Sample equivalent to 20 mg of Granisetron was placed into 50 ml volumetric flask added 30 ml of diluent and sonicated for 20 min with intermediate shaking at a temperature not more than 20 °C and then added respective degradant (Acid, Alkali, Oxidant) and performed the stress study. Samples were neutralized after degradation and then diluted to the volume with diluent and injected to verify the stability indicating power of the analytical method. Stress conditions under which the study was performed, the amount of Granisetron remains, %impurities generated and mass balance results were tabulated.

Table 7.2 : Stress condition and its results

Sr.No	Stress condition	Drug remained (%)	Impurities (%)	Mass Balance
1.	2N HCl_50 °C_3 h	98.9	1.1	97.6
2.	1 N NaOH_50 °C_3 h	99.3	0.9	96.8
3.	10 % H2O2_50 °C_2 h	81.9	18.1	95.2
4.	105 °C_48 h	98.7	1.6	98.9
5.	Photolytic stability	98.5	1.4	99.4

7.3 PRECISION:

According to the ICH, precision should be performed at three different levels: repeatability, intermediate precision, and reproducibility. Repeatability involves analysis of replicates by conducting precision study over a short period of time using same equipment. Intermediate precision involves precision study within laboratory variations due to random events such as different days, analysts, an equipments etc.

7.3.1 System Precision:

Sample solution (200 µg/mL) was injected in six replicate injections to check the Relative Standard Deviation (% RSD) for finding the precision of the system to be used for validation.

Acceptance Criteria: RSD should not be more than 2.0 %.

Table 7.3: System Precision data for Granisetron

Injection	Area of Standard
1.	5306969
2.	5316611
3.	5324700
4.	5330801
5.	5333776
Avg	**5322572**
SD	**10917**
% RSD	0.21

The % RSD of peak area for Granisetron was found to be 0.21 which is below 2.0% indicates that the system gives precise result.

7.3.2 Method Precision:

Precision of the analytical method is the closeness agreement for a series of measurement from multiple samplings as mentioned in ICH Q2 (R1). As per the guidelines, method precision and intermediate precision were analysed on the homogeneous sample and the % RSD for precision and intermediate precision was calculated and reported.

Method precision was performed by preparing six different samples as per the test concentration and analysed as per the developed method. The % Assay was calculated by using the area and concentration of the sample against 200 ppm standard sample. The % RSD was calculated for these six samples and was foundto be 0.32. Intermediate precision was performed on Homogeneous samples on a different day using different UPLC system and UPLC column by a different analyst. The % Assay was calculated for these six samples and % RSD was calculated. The cumulative % RSD was calculated for the twelve samples and the % RSD was found to be 0.3 and the average Assay was found to be 98.5.

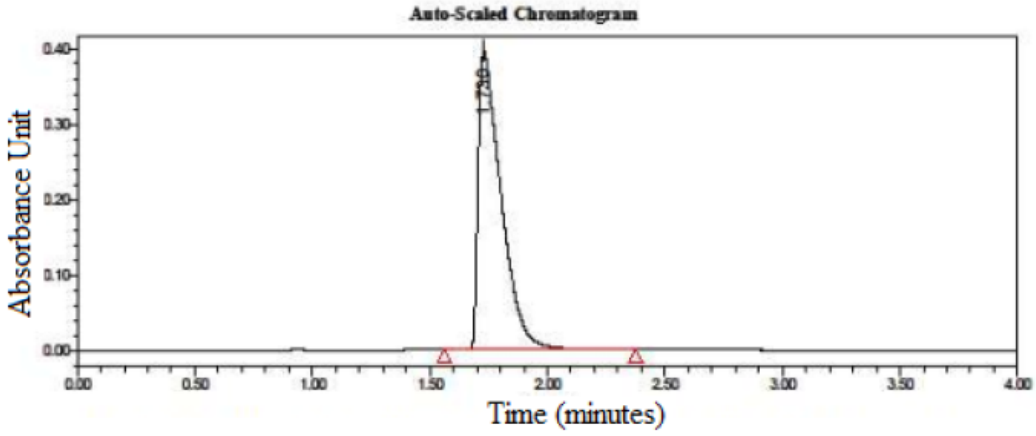

Fig. 7.5: Chromatogram of Method precision sample

The % RSD of method precision was found to be 0.40 % for Granisetron. Therefore, the UPLC method for the determination of assay for Granisetron in formulation was found to be precise

7.4 ACCURACY:

Accuracy of the analytical method is the closeness of agreement between the true value and experimental value. Accuracy of the method was performed at 3 different levels ranging from 50 % to 150 % of the Assay concentration level. The % recovery was calculated the % Assay at each level of spiked sample with as such sample.

Recovery studies were performed to judge the accuracy of the developed method. The study was evaluated by spiking the known quantity of Granisetron at various levels on the placebo. From the amount of Granisetron found the % recovery was calculated. Recovery was performed at different levels ranging from 50 % to 150 % of the specification level. The % recovery of each level was found to be within the acceptance criteria of 98 % to 102 %. So the method is accurate for the determination of Granisetron

Table 7.4 : Accuracy Table (Recovery of Granisetron)

Levels	% Recovery	Mean % recovery	% RSD
50 %	100.1	99.8	0.30
	99.8		
	99.5		
100 %	100.9	100.0	0.90
	99.1		
	100.1		
150 %	100.9	100.9	0.35
	101.2		
	100.5		

7.5 LINEARITY:

Linearity of the developed method was evaluated by injecting the Granisetron at five different levels ranging from 10.22 to 30.55 µg/ml. The concentrations ranged from 50% to 150 % of sample concentration. The respective peak area was recorded and plotted against standard concentration and the graph resulted in straight line. The correlation coefficient, slope, intercept and % Y-intercept values were calculated and tabulated for Granisetron and. From this data it was clearly indicated that the method is linear over the range of 50 % to 150 %. More over the method is sensitive for detection of Granisetron and low levels in both bulk and finished formulations.

Table 7.5 : Linearity of detector response Granisetron

Levels (%)	Concentration (µg/ml)	Area response of Granisetron
50	10.220	2674567
75	15.340	3986850
100	20.350	5329134
125	25.120	6651417
150	30.550	7983701
Correlation coefficient		**0.999**

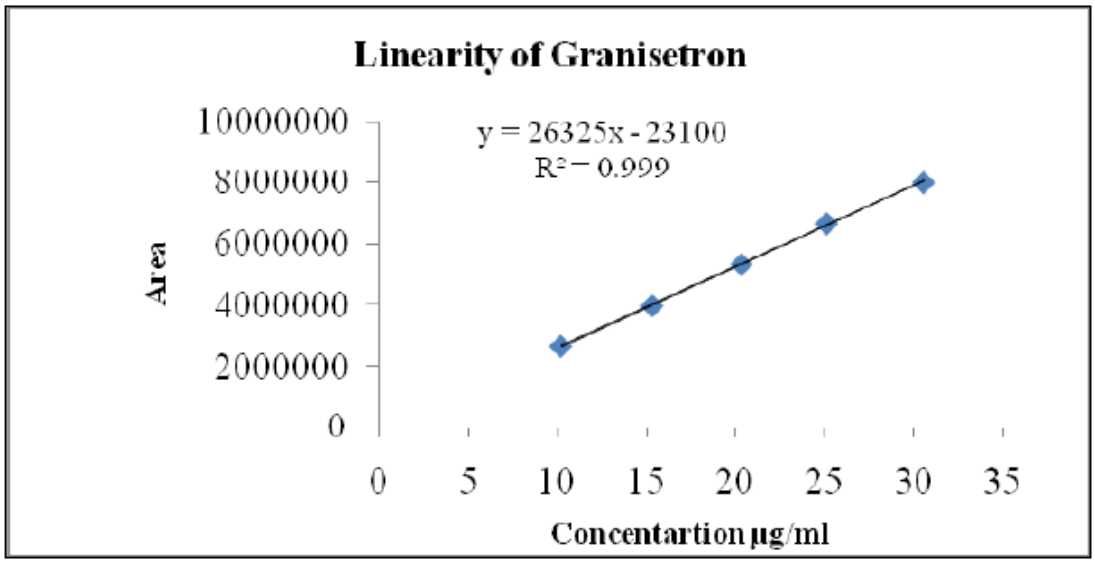

Fig. 7.6: linearity of detector response for Granisetron

The method for the estimation of Granisetron was found to be linear and the correlation coefficient was found to be 0.999. The method was found to be linear in the range of 10.22 to 30.55 µg/ml.

7.6 Range:

The range of analytical method is the interval between the upper and lower levels of analyte that has been demonstrated to be concluded that the method is linear, precise and accurate between 10.22 to 30.55 µg/ml.

7.7 ROBUSTNESS:

For the UPLC method, the robustness was determined by the analysis of the samples under a variety of conditions such as: -

7.7.1 Flow Rate Variation:

Standard, check standard and sample solutions were prepared as per the test method and injected into UPLC system with flow rates of 0.2mL/min and 0.6mL/min. System suitability parameters were evaluated as per test method and the % assay results were calculated.

Table 7.6 : System Suitability Data for Flow Rate Variation

System suitability parameters	Optimized Method (0.4 mL/min)	Flow plus (0.6 mL/min)	Flow minus (0.2 mL/min)
The % RSD for area of Granisetron for five replicate injections of standard solution	0.21	0.20	0.15
The theoretical plates for Granisetron	15193	16862	13168
The tailing factor for Granisetron	1.18	1.11	1.35
Retention time of Granisetron (min)	1.75	1.22	2.13

7.7.2 Column Oven Temperature Variation:

Standard solution was prepared as per the test method and injected into UPLC system with column oven temperature of 35 °C and 45 °C. System suitability parameters were evaluated as per test method and the % assay results were calculated.

Table 7.7 : System Suitability Data for Column Temperature Variation

System suitability parameters	Optimized Method (40 °C)	column minus (35 °C)	Column Plus (45 °C)
The % RSD for area of	0.21	0.37	0.45

Granisetron for five replicate injections of standard solution			
The theoretical plates for Granisetron	15193	14573	14648
The tailing factor for Granisetron	1.18	1.24	1.08
Retention time of Granisetron (min)	1.75	1.95	1.54

7.7.3 Effect of pH Variation in Mobile Phase:

Standard solution was prepared as per the test method and injected into UPLC system with two different mobile phases having buffers pH 6.3 and pH 6.7. System suitability parameters were evaluated as per test method and the % assay results were calculated.

Table 7.8 : System Suitability Data for pH Variation

System suitability parameters	Optimized Method (6.50)	pH plus (6.70)	pH minus (6.30)
The % RSD for area of Granisetron for five replicate injections of standard solution	0.21	0.21 0.47	0.53
The theoretical plates for Granisetron	15193	13587	14785
The tailing factor for Granisetron	1.18	1.25	1.05
Retention time of Granisetron (min)	1.75	1.89	1.69

Table 7.9 : Robustness Parameters Results

Chromatographic conditions	Normal	Variation	Assay	% RSD
pH (mobile phase)	6.50	6.3	99.9	0.3
		6.7	100.2	0.2
Flow rate (mL/min)	0.4	0.2	100.4	0.5
		0.6	99.9	0.1
Column Temperature (ºC) Acquity BEHC18 (1.7 µm, 100 mm *2.1 mm)	40	35	100.4	0.6
		45	100.8	1.2

The % RSD in the various parameters was found to be less than 2 %.

7.8 STABILITY IN ANALYTICAL SOLUTION:

Standard and sample solutions of Granisetron were kept at Refrigerator (2-8 °C) and room temperature (at 25 º C). It was analyzed initially and at different time intervals till 48 h.

% Assay value should be NMT 2.0 %.

Table 7.10 : Summary Data for Stability in Analytical Solution at Refrigerator

	Sample 1		Sample 2	
Time	**Assay(%)**	**Deviation from initial (%)**	**Assay (%)**	**Deviation from initial (%)**
Initial	98.9	-	99.9	-
Day-1	99.6	0.7	99.6	0.3
Day-2	100.6	1.7	99.9	0.0

Table 7.11 : Summary Data for Stability in Analytical Solution at room

	Sample 1		Sample 2	
Time	**Assay(%)**	**Deviation from initial (%)**	**Assay (%)**	**Deviation from initial (%)**
Initial	98.9	-	99.9	-
Day-1	101.0	2.1	101.9	2.0
Day-2	101.5	2.6	102.3	2.4

The results indicate that the system suitability solution and sample solution are stable up to 48 h at refrigerator unstable at room temperature.

7.9 LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

For the present developed UPLC method Limit of Detection was found to be 0.25 µg/mL and Limit of Quantification was found to be 0.77 µg/mL for Granisetron. LOD and LOQ were determined based on signal to noise ratio.

8.0. SUMMARY

A simple, accurate and reproducible reverse phase UPLC method was developed for the estimation of Granisetron in bulk drugs and formulations. The optimized method consists of mobile phase pH 6.5 ammonium acetate buffer and Acetonitrile in gradient elution mode with a run time of 4 min and a flow rate of 0.4 ml/min. UV detection was carried out at a wavelength of 305 nm with an injection volume of 2 µL. Acquity BEHC18 (1.7 µm, 100 mm *2.1 mm) column. The retention time of Granisetron was found to be 1.75 min. The developed method was validated as per ICH Q2A (R1) guideline. The proposed UPLC method was linear over the range of 10.22-30.50 µg/ml, the correlation coefficient was found to be 0.999. Relative standard deviation for method precision was found to be 0.32 and intermediate precision was found to be 0.34. Limit of Detection was found to be 0.25 µg/ml and Limit of Quantification was found to be 0.77 µg/ml respectively.

9.0. CONCLUSION:

The new UPLC method developed and validated for determination of Granisetron pharmaceutical dosage forms and assured the satisfactory precision and accuracy and also determining lower concentration of drug in its solid dosage form by RP-UPLC method. The method was found to be simple, accurate, economical and rapid and they can be applied for routine analysis in laboratories and is suitable for the quality control of the raw materials, formulations, dissolution studies and can be employed for bioequivalence studies for the same formulation.

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